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The Journal of Laboratory and Clinical Medicine

VOL. 45 JUNE, 1955 No. 6

CLINICAL AND EXPERIMENTAL

TISSUE BINDING OF HYDROCORTISONE*

MARVIN E. LEVIN, M.D.,** WILLIAM H. DAUGHADAY, M.D., AND RITA BREMER, M.D.
ST. LOUIS, MO.

CORTISONE and hydrocortisone rapidly leave the blood of man and animals following intravenous administration.[1-3] The role of the liver in removing corticosteroids from the circulation and affecting their chemical degradation has been established by many workers.[4-6] However, it is difficult to explain the clearance of steroid from the blood by degradation alone.

In considering other fates of hydrocortisone within the body, it would seem unlikely that the steroid distributes equally in body water. In the first place, hydrocortisone, even at high blood levels, does not diffuse into normal cerebrospinal fluid.[7] Moreover, in the hepatectomized rat, a preparation which degrades hydrocortisone very slowly, the calculated apparent volume of steroid distribution exceeds total body water.[3]

It would appear more likely that the distribution of hydrocortisone in the body is to a great degree determined by the ability of certain tissue constituents to bind steroid rapidly. That this may be due to the protein nature of the tissue is suggested by the high degree of binding to serum albumin of other steroids (testosterone and progesterone).[8] The measurements of steroid

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solubilities in protein solutions as shown by Eik-Nes and associates[9] have also indicated that hydrocortisone is bound to a considerable extent by collagen and serum albumin.

To elucidate more fully the fate of hydrocortisone within the body, a study has been conducted of the combination of hydrocortisone with rat liver homogenates and tissue slices.

METHODS

Animals.—Male rats of the Sprague-Dawley strain weighing between 200 and 250 grams were fed Purina laboratory chow during the period of these experiments.

Disappearance of Hydrocortisone From the Blood of Intact Rats.—A standard dose of 0.2 mg. of hydrocortisone per 100 grams of rat in a solution containing 1 per cent alcohol and 5 per cent glucose was injected into the tail vein. At intervals from 5 to 60 minutes the animals were decapitated and the blood was collected in 15 ml. graduated tubes. The volume was noted and the blood 17 OH corticosteroids were measured by a modification of the procedure of Nelson and Samuels.[10]

Incubation of Hydrocortisone With Rat Tissue Slices.—Incubations were carried out in 50 ml. Erlenmeyer flasks using 3 ml. of Krebs's phosphosaline buffer containing 250 to 1,000 μg of hydrocortisone prepared according to the directions of Schneider.[4] Slices of liver, kidney, spleen, and fat were cut with a Stadie-Riggs microtome. Hemidiaphragms were added directly to the incubation flask. Two hundred to 250 mg. of tissue was added to each flask. The flasks were shaken at a rate of 100 cycles per minute in a water bath at 37° and 0° C.

The experiments were performed in two ways. In Method I, the hydrocortisone disappearing from the medium and that which could be recovered from the total contents of the flask (medium plus tissue) were measured separately. This was carried out by pipetting 1 ml. of the medium into 2 per cent sodium chloride and extracting with ethyl acetate. Aliquots of the ethyl acetate extract were evaporated in centrifuge tubes and the hydrocortisone determined by the Porter-Silber reaction.[11] Immediately after sampling the medium the reaction in the flasks was stopped by the addition of 30 ml. of acetone. The acetone extract was filtered into a separatory funnel through a Whatman No. 2 filter paper. The tissue slices were then thoroughly ground with washed sand, extracted with four 10 ml. aliquots of hot acetone, and filtered into the same separatory funnel. Forty milliliters of water was added to the acetone and the extract was shaken with 50 ml. of hexane twice. The hexane washes were combined and back extracted with 50 ml. of 50 per cent aqueous acetone, the acetone fractions were placed in a boiling flask and the acetone was removed at 45° C. under reduced pressure. The residual aqueous solution was transferred to a separatory funnel and extracted 3 times with 30 ml. of ethyl acetate. The ethyl acetate extracts were combined, dried over sodium sulfate, and diluted to 100 ml. Aliquots were removed for analysis using the Porter-Silber reaction.

In Method II the hydrocortisone which disappeared from the medium and that which remained in the tissue slices after washing with steroid-free buffer were measured. This was done by decanting the medium into a separatory funnel at the conclusion of the incubation. The slices were then vigorously washed 4 times with 10 ml. of steroid-free buffer. The incubation medium and the buffer washes were combined and extracted with ethyl acetate. Hydrocortisone was determined again by the Porter-Silber reaction. The residual steroid in the tissue slices was measured as in Method I after extracting the ground slices with hot acetone.

Dialysis of Rat Liver Homogenate Against Buffer Containing Hydrocortisone.—Five grams of rat liver was ground in a Potter Elvehjem glass homogenizer with 15 ml. of Krebs's phosphosaline buffer. Three milliliter aliquots of the homogenate were placed in Visking Cellophane bags, and 2 small Pyrex beads were added to each bag to ensure mixing. The bags were next dialyzed for 3 hours in a large volume of Krebs's buffer and

then transferred to test tubes containing 10 ml. of Krebs's buffer having a concentration of 150 μg of hydrocortisone per milliliter. The tubes were inverted every 8 seconds for 24 hours on a motor-driven disk at 4° C.

At the conclusion of the dialysis the contents of the bag and the surrounding fluid were separately analyzed for protein by the biuret method after precipitation with 10 per cent trichloroacetic acid,[12] for water by weighing before and after desiccation, and for hydrocortisone by the method described previously.

Each point in Figs. 1 through 6 is the average result obtained from 3 to 14 separate incubation flasks. Ninety to 100 per cent of added hydrocortisone was recovered using the extraction conditions described. Extraction with chloroform led to erratic and occasionally large losses of hydrocortisone. Precipitation of the proteins with ethanol using different conditions did not yield good recoveries of steroid from the tissue slices.

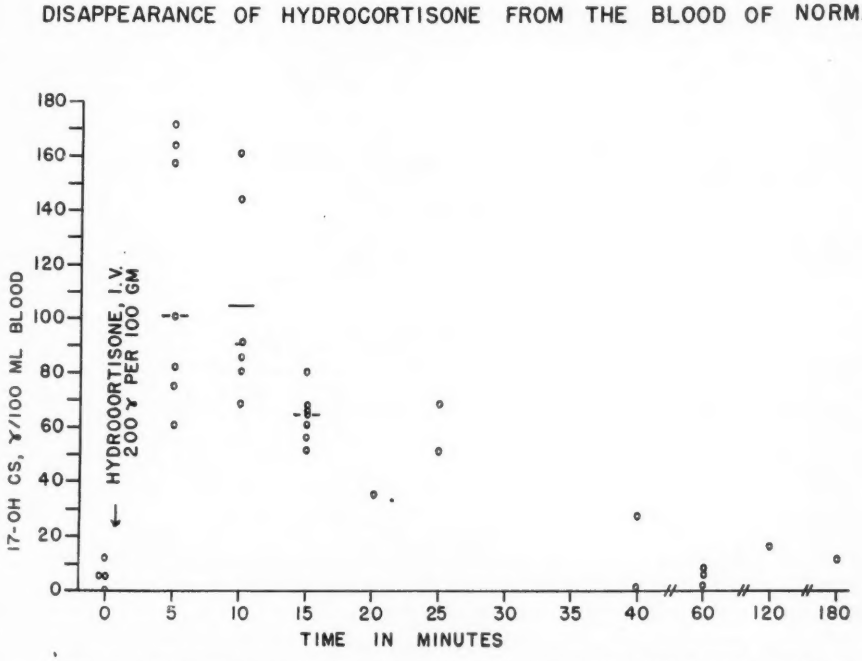

Fig. 1.—Disappearance rate of hydrocortisone from the blood of normal rats following the intravenous administration of 0.2 mg. of hydrocortisone per 100 grams of rat. Only 2 per cent of the administered dose remained within the blood after 15 minutes.

RESULTS

The rapid disappearance of hydrocortisone from the blood of normal rats after intravenous injection has been confirmed (Fig. 1). Only 2 per cent of the administered dose remained within the blood after 15 minutes.

Analyses carried out according to Method I (see Methods) permitted the simultaneous measuring of the degradation of the Porter-Silber reactive steroid (PSRS) and its combination with liver slices (total PSRS in flask less PSRS in the medium). Degradation was found to occur most rapidly during the first 15 minutes (Fig. 2) but continued throughout the period of observation. Hydrocortisone combined rapidly with the liver slices; within 5 minutes a near maximal level was reached; and thereafter no appreciable increment was observed.

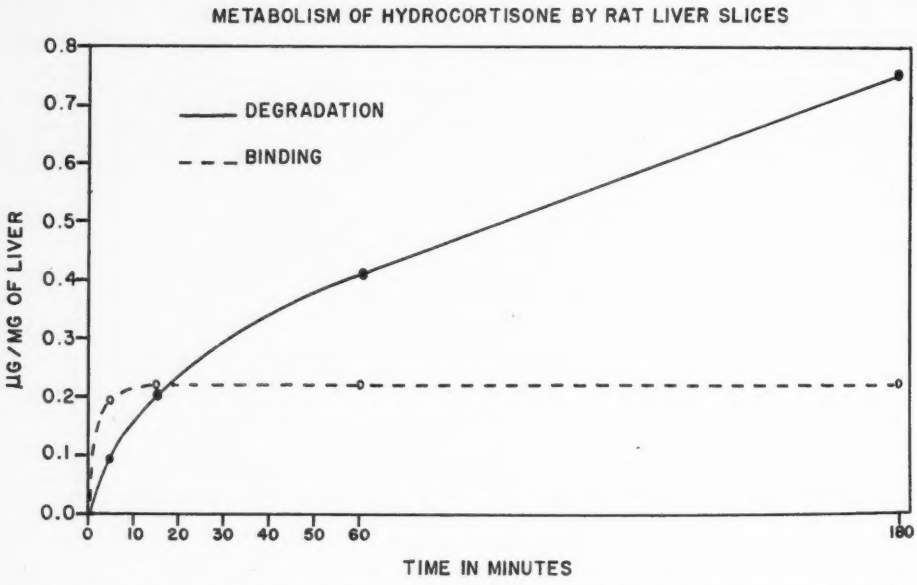

Fig. 2.—Rate of degradation and binding of hydrocortisone by 250 mg. of rat liver slices incubated at 37° with 500 µg of hydrocortisone in Krebs's phosphosaline buffer.

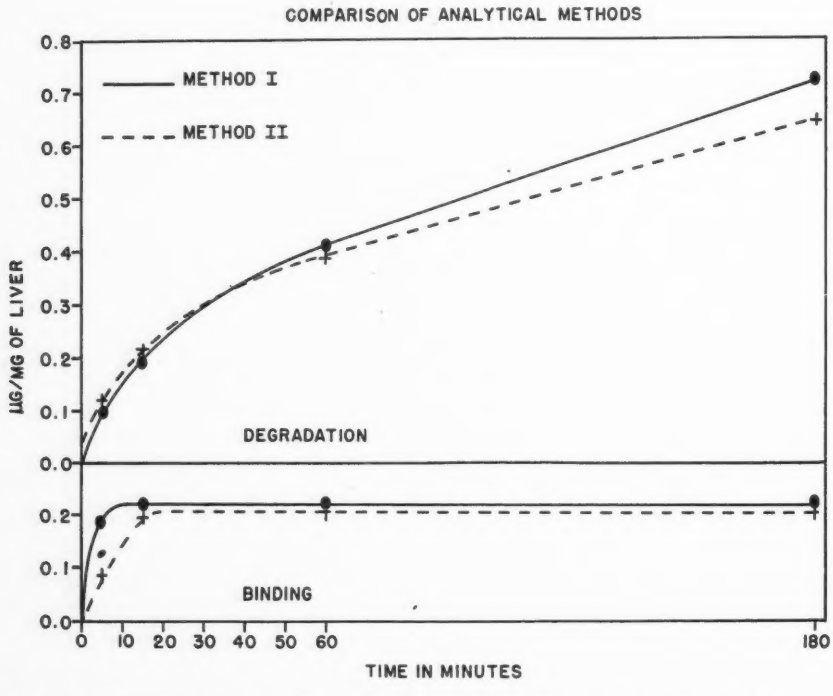

Fig. 3.—A comparison of Methods I and II (see Methods) for measuring degradation and binding of hydrocortisone by rat liver slices shows no difference in degradation. However, in Method II (extraction of the bound steroid from washed tissues) a looser binding during the first 15 minutes is suggested by noting a decreased recovery as compared to extraction from the unwashed tissues, Method I.

When the incubation of hydrocortisone with liver slices was studied using Method II (washed slices), the degradation of hydrocortisone and its binding by the slices were similar to the results obtained with Method I (Fig. 3). Some

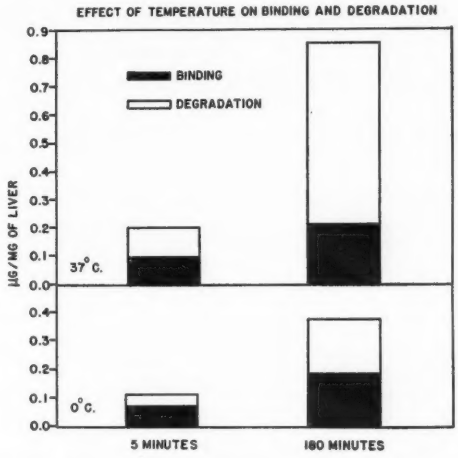

Fig. 4.—Incubation of rat liver slices with hydrocortisone at 37° and 0° C. shows essentially no alteration in the degree of binding but marked inhibition of degradation is seen during incubation at 0° C.

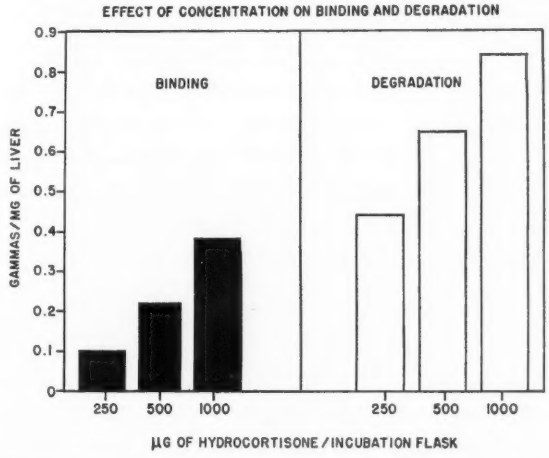

Fig. 5.—A comparison of various concentrations of hydrocortisone incubated with 250 mg. of rat liver slices shows that binding bears a 1:1 correlation with concentration. Degradation is also increased but is not directly proportional in the observed period.

difference was observed in the degree of binding during the first 5 minutes of incubation. The results obtained with Method II were lower than with Method I, suggesting that in this period hydrocortisone was more easily eluted from the liver slice. At a later stage this did not occur.

Experiments conducted with Method II disclosed that the binding of hydrocortisone by liver slices was not inhibited by performing the incubation at 4° C., although the amount of degradation was much less (Fig. 4). The degree of

steroid binding was found to be proportional to the concentration of hydrocortisone in the incubating medium (Fig. 5).

The ability of rat diaphragm, kidney, spleen, and fat to bind and degrade hydrocortisone was compared to liver (Fig. 6). The binding of hydrocortisone by all tissues tested was of the same order of magnitude except fat, which bound only negligible quantities of steroid. The degradation of hydrocortisone, however, was not proportional to the ability to bind the steroid.

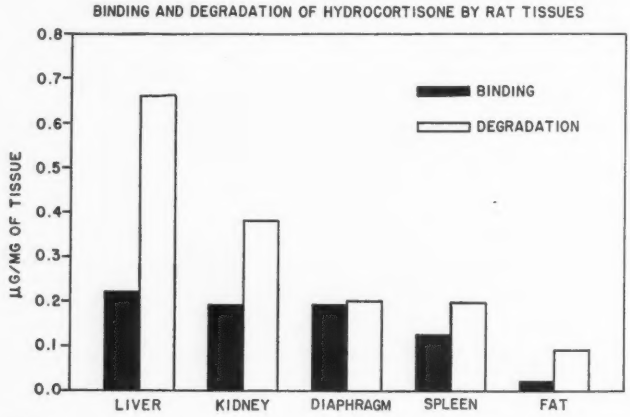

Fig. 6.—Incubation of various rat tissues with 500 μg of hydrocortisone at 37° C. reveals varying degrees of degradation from tissue to tissue, but with the exception of fat there is essentially equal binding of the steroid by all other tissues studied.

To gain additional information concerning the nature of the combination of hydrocortisone with tissue components, rat liver homogenate was dialyzed against a buffer solution containing hydrocortisone. The ability of this cell-free homogenate to bind and concentrate the steroid is clearly shown in Table I. A calculation based on the protein in the homogenate and in the liver slices indicates that the binding is of comparable magnitude.

TABLE I. BINDING OF HYDROCORTISONE BY RAT LIVER HOMOGENATE

TUBE	HYDROCORTISONE CONCENTRATION AT END OF DIALYSIS		MG. PROT./ML. OF HOMOG.	μG HYDROCORTISONE BOUND/MG. PROTEIN
	μG/ML. IN BUFFER	μG/ML. IN HOMOG.		
A	76	140	44	1.5
B	71	132	41	1.6
C	75	145	42	1.8
D (control)	98	95	0	—

Dialysis equilibrium carried out with 3 ml. of rat liver homogenate in Cellophane bags dialyzed 22 hours at 4° C. against 10 ml. of Krebs's phosphosaline buffer containing 150 μg hydrocortisone per milliliter. Total alcohol concentration was 1.5 per cent. Tube D is a control tube containing 3 ml. of steroid-free buffer in the Cellophane bag.

DISCUSSION

The extreme rapidity with which hydrocortisone disappears from the blood has suggested that in addition to its enzymatic destruction, considerable steroid combines with tissues. The results reported in this paper indicate that the

affinity of tissue proteins for hydrocortisone may contribute to its early disappearance from the blood. The fact that tissue binding of hydrocortisone was not inhibited by incubation at 0° C. suggests that binding is a physical rather than an enzymatic process. The predominance of protein rather than lipid binding is suggested by the negligible binding of hydrocortisone by slices of depot fat.

Our interpretation of the experiments with tissue slices is supported by the results of the dialysis experiments with liver homogenates. Evidence for steroid binding under these conditions appears to be conclusive.

The ability of extrahepatic tissues to bind hydrocortisone indicates that a tissue pool of hydrocortisone exists after the intravenous infusion of this steroid. Tyler and associates[13] have come to a similar conclusion on the basis of infusion studies in human beings. The rapidity with which isotopically labeled steroids reach the liver and are excreted in the bile[14] suggests that the steroids in nonhepatic tissues are largely loosely bound and in equilibrium with the steroid in the plasma. Thus, as the plasma level of hydrocortisone falls due to hepatic removal, hydrocortisone presumably re-enters the circulation and is subsequently metabolized by the liver. It may be speculated that the affinities of certain sites on the tissue proteins are greater than others and that a protein steroid complex is the physiologically active form of the hormone.

Certain features of the in vitro metabolism of hydrocortisone by tissue slices observed in these experiments differ from the known metabolism of this steroid. Liver slices did not bind significantly more hydrocortisone than did several other tissues; yet in the intact animal the concentration of tritium is much higher in liver than other tissues after the administration of tritium-labeled cortisone.[2] It is possible that the disruption of the biliary drainage system prevents greater accumulation of hydrocortisone by liver slices. It is also worthy of note that the hepatectomized rat degrades hydrocortisone very slowly, if at all.[3] However, the degradative activity of nonhepatic tissues in vitro is significant, and when the total mass of such tissue is considered, the destruction of hydrocortisone outside the liver could be considerable.

SUMMARY

Rat tissue slices have been incubated with hydrocortisone in phosphosaline buffer. The amount of hydrocortisone degraded and that which combined with the slices without loss of the Porter-Silber reactive group was measured in two ways. The results show that hydrocortisone was bound rapidly by liver slices at both 0° and 37° C. Diaphragm, kidney, and spleen also bound hydrocortisone but had substantially less ability to degrade the steroid. Only fat appeared not to bind hydrocortisone. A homogenate prepared from rat liver bound 1.5 to 1.8 μg of hydrocortisone per milligram of protein.

It is suggested that the ability of tissue proteins to bind hydrocortisone contributes to its rapid disappearance following intravenous injection. The possibility that hydrocortisone acts physiologically as a protein-steroid complex is suggested.

Grateful acknowledgment is made to Merck & Co., Inc., for generously supplying the hydrocortisone used in these studies.

We are indebted to Carolyn Johnston and Carolyn Hartnett for technical assistance.

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A STUDY OF METHODS FOR THE PREDICTION OF PLASMA VOLUME

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BECAUSE of the wide variation in plasma volume of healthy individuals, there are no satisfactory normal values or standards of reference. For this reason, the clinical application of plasma volume determination in disease states has been somewhat limited. This is especially true whenever significant small changes in plasma volume have been sought. It was felt that a contributing factor to the wide range of normal values might be the variation in vascularity of various body tissues, most notably the difference between fat and muscle tissue. If this source of variation in relating total volume to physical measurement could be standardized, a more accurate calculation of expected plasma volume might be available for individual cases, and would, therefore, permit better evaluation of small variations in plasma or blood volume.

A second source of error in the interpretation of plasma or blood volume has been emphasized by Funkhouser and Pritchard.[1] There is a mathematical inaccuracy in establishing a direct ratio of body measurement to plasma or blood volume. They pointed out that unless the regression line relating volume to body size passes through the origin, it is mathematically incorrect to assume a direct ratio between volume and linear measurement. The purpose of this paper, therefore, has been (1) to determine the difference of two plasma volume determinations performed in the same person at a two-week interval and (2) to re-examine the possibility of selecting a common denominator to which plasma volume could be related.

PROCEDURE

A. *Selection of Patients.*—Group I consisted of 13 subjects on whom plasma volume determinations were carried out by I^{131}-tagged human albumin and Evans blue dye on two occasions at two-week intervals. Fifty-one subjects of both sexes were studied in Group II, and estimation of specific gravity and total body water by antipyrine as well as plasma volume by both I^{131}-tagged albumin and Evans blue dye was made. The subjects were chosen at random from hospital personnel and were all in good health. All body types were included, but no attempt was made to concentrate on extremes.

B. *Method of Study.*—The subjects were studied in a fasting state after a short period of rest. A control blood sample was drawn without stasis and a known amount of Evans blue dye administered intravenously. Blood samples were taken from the opposite arm at exactly 10 minutes, as described by Gregersen.[2] A known amount of radioactive iodinated human serum albumin was given through the needle used for the 10-minute Evans blue dye sample, and blood was taken from the original arm at 10 and 20 minutes.

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in Group II, 1 Gm. of antipyrine* was administered from a calibrated syringe through the needle used for the 20-minute I^{131} blood sample. Specimens were then drawn at 2, 3, and 5 hours after the initial injection for antipyrine determinations. Height and weight were taken and the Group II subjects were weighed, completely submerged, in a tank of water after a maximum expiration. Residual air studies were done at a later date.

TABLE I. PLASMA VOLUME VALUES FOR GROUP I SUBJECTS. A TWO-WEEK INTERVAL EXISTS BETWEEN FIRST AND SECOND DETERMINATIONS

SUBJECT	1ST DETERMINATION	2ND DETERMINATION	DIFFERENCE
	I^{131} Albumin		
SCH	3,886	3,383	503
MAN	3,564	3,750	186
MER	3,827	3,750	77
LON	3,655	3,734	79
TOB	2,852	2,732	120
TOJ	3,263	3,003	260
KRA	2,330	3,135	805
WRI	3,191	3,446	255
MOO	3,589	3,329	260
JOH	3,041	3,538	497
Mean	3,319	3,380	
Difference between means		61	
	Evans Blue		
SCH	2,800	2,800	0
BOS	2,520	2,300	220
LON	3,652	3,370	282
TOB	2,975	3,210	235
TOJ	3,190	3,430	240
KRA	2,995	2,920	75
WRI	3,003	3,220	217
JOH	2,867	3,250	383
COY	3,105	3,080	25
Mean	2,710	2,758	
Difference between means		48	

Methods of Measurement.—Evans blue dye determinations were carried out on a Beckman DU spectrophotometer at 620 mμ by a method similar to that of Gibson,[3] using the patient's blank serum sample, a dyed standard serum sample, and a 10-minute post-injection sample. Heparinized plasma samples were counted for radioactivity with a conventional Geiger-Müller tube for a minimum of 5,000 counts according to the method of Storaasli.[4] Samples were counted before and after being air dried. The standards used were prepared and counted in the same manner. About one-half of the samples were also counted as whole blood with a scintillation counter by placing the whole-blood samples in a Klett tube and inserting them into a shielded chamber at a standard distance from the iodide crystal, as described by McIntyre.[5] Antipyrine determinations were carried out according to the method of Brodie and associates.[6, 7] The samples were read in a Beckman DU spectrophotometer using the hydrogen discharge lamp at 350 mμ. Concentration was plotted against time on semilog paper and extrapolated to zero time. Total body water was then figured from the following formula:

$$\frac{\text{1 Gm. antipyrine}}{\text{gamma/ml. in plasma}}.$$

This value was then corrected for 8 per cent solids and the final figure used as the value for total body water.

*We are indebted to Dr. Kenneth G. Kohlstaedt of Eli Lilly & Company, who generously donated the antipyrine used in this study.

Residual air studies for determination of specific gravity were carried out by the open circuit method of Darling, Cournand, and Richards[8] and the analysis for CO_2 and O_2 done by the method of Van Slyke and Sendroy.[9]

From the figures for specific gravity and total body water (antipyrine), body fat and lean body mass were estimated. The formula of Rathbun and Pace[10] was used with the specific gravity data:

$$\text{per cent body fat} = 100\,\frac{(5.548 - 5.044)}{\text{specific gravity}}.$$

The formula of Osserman and co-workers[11] was used with antipyrine body water values:

$$\text{per cent body fat} = 100\,\frac{\text{(body weight} - \frac{\text{weight body water)}}{0.718}}{\text{body weight}}.$$

Lean body mass was estimated by subtracting the weight of fat from total weight.

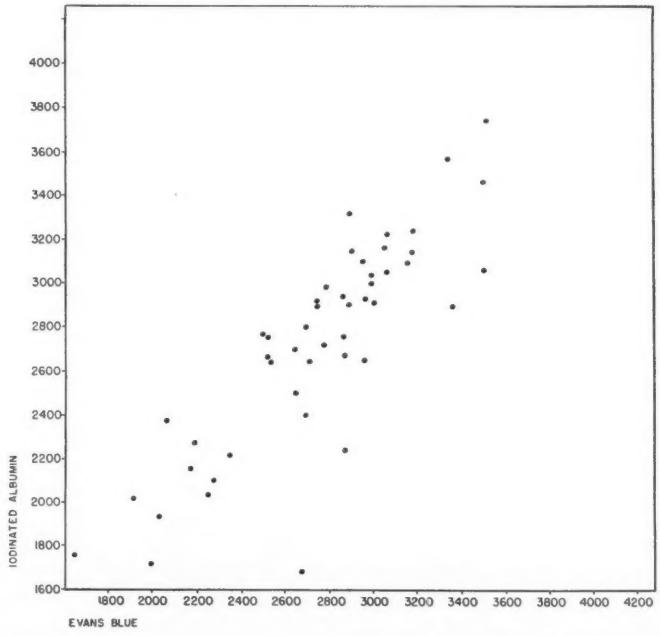

Fig. 1.—Plasma volume Evans blue compared with plasma volume iodinated albumin (Geiger) in cubic centimeters.

RESULTS

The mean values for plasma volumes in subjects in Group I showed close agreement between the first and second determinations (Table I). In order to determine the standard deviation of the distribution of plasma volume determinations in one individual at different times, the individual means and deviations from the mean were determined. Standard deviations of 156 ml. for the Evans blue dye method and 289 ml. for the iodinated human serum albumin method were found. The high standard deviation for the isotope method is the result of one striking variation as seen in Table I. If this value were excluded, the standard deviation for this method would be only 173 ml.

Since this work has been completed, several authors[12, 13] have indicated that Evans blue dye and iodine-tagged human serum albumin give comparable values for plasma volume. Our results also showed a close correlation (Fig. 1). The slope of the line of regression for Evans blue dye against weight

TABLE II. PLASMA AND BLOOD VOLUME VALUES ON 51 SUBJECTS (GROUP II)

NO.	CASE	WEIGHT (KG.)	SURF. AREA (M^2)	BODY WATER ANTI-PYRINE (ML.)	BODY WATER SPEC. GR. (ML.)	PLASMA VOL. EVANS BLUE (ML.)	PLASMA VOL. I^{131} (DRY) (ML.)	HEMATO-CRIT (%)	BLOOD VOL. EVANS BLUE (ML.)	BLOOD VOL. SCINTIL-LATION I^{131} (ML.)
1	HK	71.93	1.83	45,280	46,700	2,912				
2	SI	82.27	2.07	41,805	42,850	2,739	3,015	47	5,168	5,570
3	PH	78.97	1.99	48,960	45,900	3,490	3,877	42	5,980	6,450
4	JB	71.02	1.79	37,480		2,950	3,135	45	5,393	5,330
5	HMc	65.20	1.74	44,360	43,050	2,497	2,808	45	4,556	5,600
6	JS	74.40	1.97	49,400	42,800	3,180	3,086	44	5,932	
7	BB	81.80	1.97	46,250	45,400	2,990	3,096	48	5,761	
8	RF	74.38	1.95	52,173	46,900	2,740	2,859	45	4,981	6,420
9	RW	75.10	1.96	39,525	39,800	3,060	3,388	41	5,204	5,170
10	HM	85.45	1.91	39,525		3,360	3,085	46	6,269	
11	WI	73.30	1.94	46,253	40,650	2,780	2,825	47	5,225	
12	IU	68.18	1.78	44,365	36,830	2,340	2,347	42	4,048	
13	JDe	76.80	1.93	50,555	46,990		3,172	42		
14	DM	78.97	2.00	42,625	39,100	3,001	3,047	46	5,547	4,970
15	JC	77.84	1.91	42,625		3,500	3,078	45	6,329	
16	JK	77.50	1.96	45,289	54,300	2,960	2,799	48	5,670	
17	RS	63.70	1.85	40,257	36,300	2,870	3,099	50	5,740	5,450
18	JMc	59.77	1.67	33,444		2,530	2,742	43	4,423	5,230
19	GT	70.68	1.81	51,030	42,780	2,000	1,631	48	3,135	3,250
20	JP	82.15	2.06	50,555		3,495	3,587	42	6,067	6,170
21	WW	81.13	1.97	38,819		2,513	2,861	42	4,325	5,090
22	CB	63.40	1.70			2,520	2,678	50	5,009	4,800
23	RL	78.86	2.00	51,759	45,700	2,894		44	5,131	
24	WD	77.04	1.93	37,480	42,300	2,866	2,949	47	5,447	5,070
25	EB	63.75	1.73	37,872		2,654	2,566	50	5,340	4,730
26	GS	71.13	1.80	45,858	45,600	2,960	2,911	47	5,584	5,070
27	AB	82.15	2.07	47,882	49,300		4,491	41		
28	FT	67.61	1.84	44,365		2,685	2,737	48	5,223	5,160
29	DH	78.18	1.96	44,730		2,900	3,258	44	5,206	5,930
30	RW	77.72	1.96	44,915	42,700	2,990	3,157	45	5,476	5,290
31	RP	72.04	1.87	43,478	37,800	3,060	3,200	45	5,543	6,000
32	BL	85.90	2.11	47,258		3,340	3,630	44	5,943	6,350
33	JB	70.11	1.82	39,525		3,050	3,149	40	5,041	5,050
34	PT	74.54	1.80	43,478		2,675	2,313	45	4,863	
35	LB	60.45	1.67	35,063		2,890	3,100	41	4,941	5,670
36	LE	56.54	1.57		26,300	2,070	2,461	44	3,723	4,840
37	MMe	51.02	1.52	32,445		2,168	2,234	42	3,737	3,828
38	MM	71.81	1.84	34,506		3,179	3,175	39	5,177	
39	HA	63.75	1.73	38,138		2,754	2,675	38	4,441	
40	VS	63.75	1.72	36,232	34,430	2,860	3,100	43	5,125	
41	EP	58.4	1.63	31,920		2,644	2,712	39	4,320	4,430
42	HN	39.65	1.27	26,034	21,610	1,640	1,806	34	2,496	2,880
43	AB	51.36	1.47	31,055	28,250	2,065	2,313	39	4,049	4,080
44	SC	63.86	1.73	34,838		2 712	2,669	42	4,659	
45	BV	56.60	1.67	32,445	31,090	2,190	2,209	46	4,040	
46	LG	52.95	1.56	28,984	30,720	2,250	2,018	39	3,676	
47	CW	77.38	1.87	35,872	36,320	2,690	2,239	46	5,028	
48	PC	84.88	1.87	43,828	29,560	3,165	3,219	40	5,275	
49	SM	53.97	1.50	24,980		2,270	2,216	40	3,802	
50	EM	50.00				1,910	2,133	39	3,131	
51	JS	44.54	1.32	26,191		2,026	1,954	42	3,481	

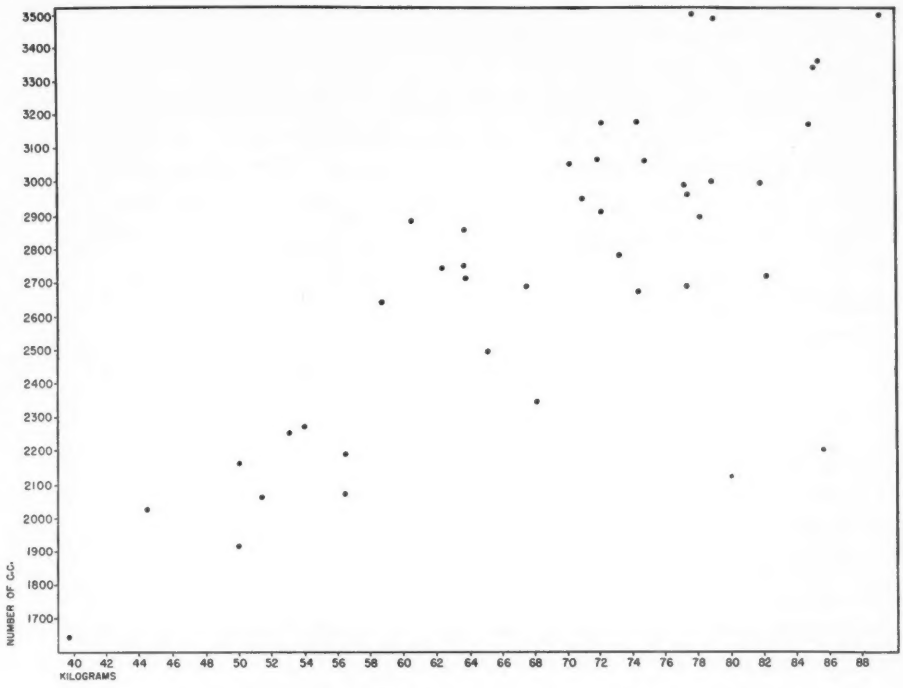

Fig. 2.—Plasma volume Evans blue against body weight.

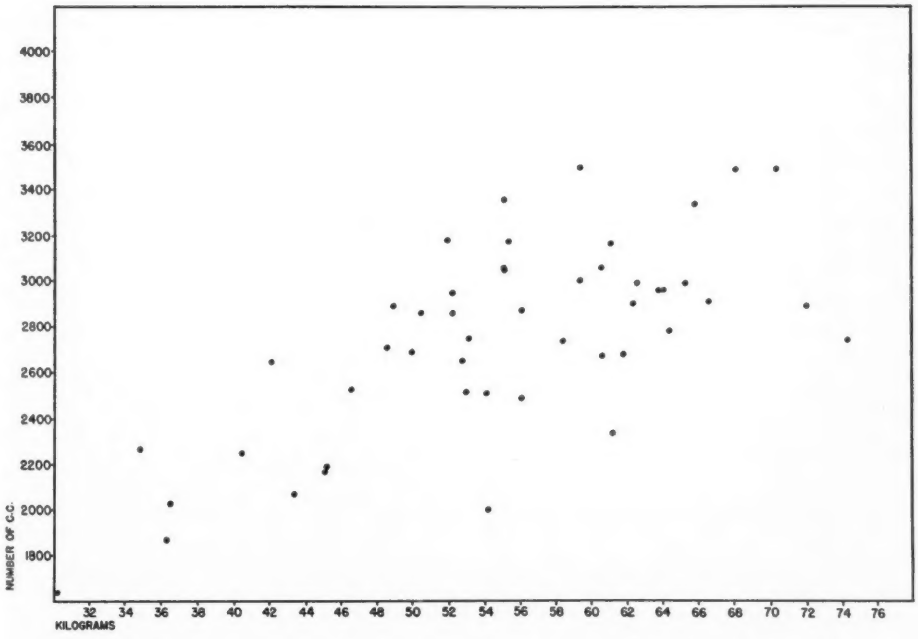

Fig. 3.—Plasma volume Evans blue against lean body weight antipyrine.

(Fig. 2) was PV = 668 ml. + 30 ml./kg. body weight and that for I^{131}-tagged albumin, PV = 620 ml. + 31 ml./kg. body weight.

TABLE III. MEAN VALUES AND STANDARD DEVIATIONS FOR PLASMA AND BLOOD VOLUMES AS RELATED TO PHYSICAL CORRELATION FACTORS. EXCEPT WHERE NOTED, VALUES ARE FOR EVANS BLUE DETERMINATIONS

	BODY WEIGHT (ML./KG.)					ANTIPYRINE BODY WATER (ML./L.)	
	PLASMA			BLOOD			
	EVANS BLUE	I^{131} DRY	I^{131} WET	EVANS BLUE	I^{131}	PLASMA	BLOOD
Mean value	40.7	41.2	39.7	71.4	73.3	50.2	90.3
St. dev.	4.4	5.5	5.3	7.3	8.8	6.5	11.5

	SPECIFIC GRAVITY BODY WATER		ANTIPYRINE LEAN BODY MASS		SPECIFIC GRAVITY LEAN BODY MASS	
	BLOOD	PLASMA	PLASMA	BLOOD	PLASMA	BLOOD
Mean value	93.3	51.1	70.0	126.7	71.3	129.5
	5.6	6.6	9.3	15.1	10.2	17.3

The average blood volume per kilogram of body weight in 28 subjects in whom blood volume was calculated by both Evans blue plasma volume and hematocrit, and by counting whole blood with a scintillation counter, was 72 ml. per kilogram for the hematocrit, plasma volume method and 73 ml. per kilogram counting the radioactivity of the whole blood.

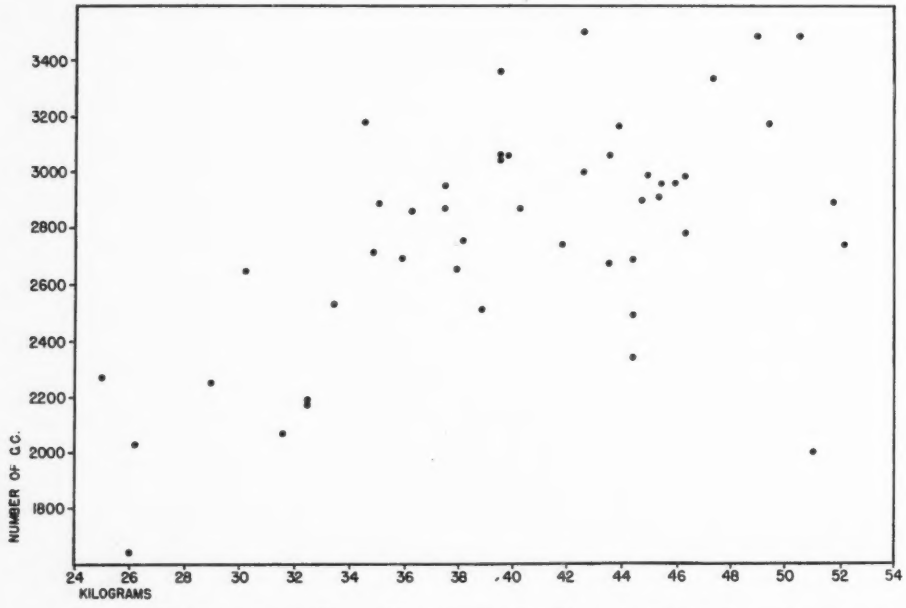

Fig. 4.—Plasma volume Evans blue compared with antipyrine body water weight.

Plasma samples containing radioactive iodine were counted as both wet and dry samples in order to evaluate the accuracy of doing rapid determinations on fresh samples. The average of 39.7 ml. per kilogram for wet counted

samples was not significantly lower than the 41.2 ml. per kilogram obtained with dry samples. Data are presented in Table II.

Inasmuch as both the dye and labeled albumin methods provided similar results, the Evans blue dye values have been arbitrarily chosen for relating plasma volume to body measurement (Table III). In the accompanying figures, therefore, all plasma volumes were those determined by Evans blue dye.

Plasma volume per kilogram of lean body mass was 50.2 ml. per kilogram with a standard deviation of 6.5 ml. (Fig. 3). Almost identical values are seen when lean body mass is determined by the specific gravity method. Plasma volume per liter of antipyrine space (Fig. 4) is 70 ml. per liter with a standard deviation of 9.3 ml. The value for body water determined from specific gravity is 71 ml. per liter with a standard deviation of 10.1 ml.

DISCUSSION

It has been shown by others, and confirmed in this study, that both the Evans blue dye and the I^{131} serum albumin tags give comparable values of plasma volume. The great advantage of using I^{131}-tagged albumin is its ease of handling for injection and blood determination if a scintillation counter can be employed. The capacity of a scintillation counter to measure the gamma radiation of I^{131} permits repeated injections of small amounts of the isotope without the complications inherent in the repeated injection of Evans blue dye. This method of counting whole blood avoids the error produced by trapped plasma in the determination of hematocrit, which has been variously estimated at between 2 and 8 per cent.[14, 15] It does not avoid the problem of small and large vessel hematocrit differences, which can be resolved only by independently tagging both the cellular and liquid components of whole blood.

Since a significant variation in plasma volume performed on the same individual exists between the two separate determinations with a standard deviation of 156 ml. in this series, it would seem unreasonable to expect to reduce this variability among a group of individuals to less than this even if an ideal method for the relation of plasma volume to body size existed.

It was emphasized that an attempt to relate plasma volume directly to surface area, height, or weight by a simple ratio is mathematically incorrect unless the line of regression constructed from this relationship passes through the origin. These data and calculations of others show a linear relationship which passes above the origin, indicating different ratios for different body builds. It is more accurate, then, to describe the slope of the line of regression as a formula for predicting plasma volume, e.g., plasma volume = 500 ml. + 30 ml. × wt. in kg., rather than the ratio, plasma volume = 40 ml. × weight in kg.

By establishing lines of regression for various body measurements such as lean body mass, body weight, body water as measured by antipyrine, height, and surface area, it was possible to determine the standard deviation of the points from that particular line and thus to determine what measurement offered the smallest variation. It was anticipated that plasma volume would

relate most accurately to lean body mass inasmuch as fat tissue is less vascular than muscle and other tissues.

The individual plasma volumes of Group II unrelated to any body modality gave a standard deviation of 413 ml. from the mean. Least squares regression lines were constructed for Group II, relating plasma volume to lean body weight, actual body weight, antipyrine space, and surface area. The three best correlations were to lean body mass with a standard deviation of 317 ml.; total body weight, with a standard deviation of 273 ml.; and surface area with a standard deviation of 267 ml. It is apparent from these values that either surface area or weight offer the best approach to predicting plasma

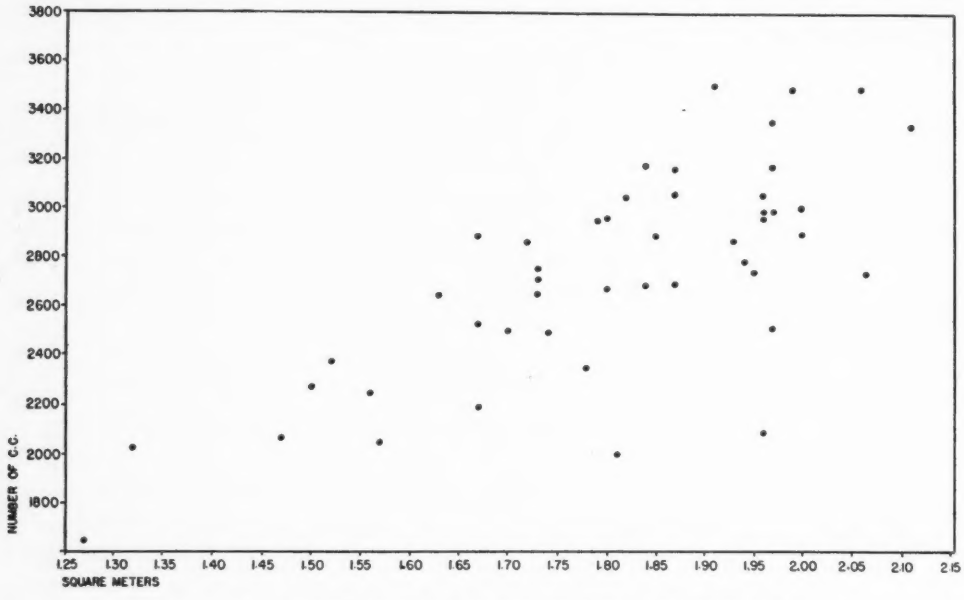

Fig. 5.—Plasma volume Evans blue compared with body surface area.

volume. A standard deviation of 267 ml. is over 100 ml. more than could be achieved in the repeated determinations carried out in Group I. The limits of variability of two-thirds the group, therefore, represent almost 20 per cent of the total volume. The slope of the line of regression for Evans blue dye plasma volume and surface area (Fig. 5) in this series was: plasma volume = 1,770 ml. × surface area M_2 − 460 ml. The slope for total body weight was: plasma volume = 668 ml. + 30 ml. × wt. in kilograms. By any current technique in use, plasma volume has definite limitations, and reliance on clinical signs and symptoms as well as other laboratory procedures should not be abandoned in favor of a plasma volume determination, which may vary considerably in any individual or within groups of individuals.

SUMMARY

Plasma volumes of two different groups of subjects were studied by similar techniques using both Evans blue dye and iodinated human serum albumin.

In Group I plasma volume estimations by two different techniques were carried out on thirteen subjects on two occasions at two-week intervals. The standard deviation in the same individual on these occasions was 156 ml. for Evans blue dye and 289 ml. for iodinated human serum albumin.

In Group II plasma volume determinations were performed on fifty normal subjects of both sexes and many body types at a single period under basal conditions. The isotope tags were assayed on both wet and dry plasma samples and from whole blood using a scintillation counter. Lean body mass and total body water were determined by body specific gravity and antipyrine methods.

From the accumulated data, the close agreement of the two methods for estimating plasma volume was confirmed. The mean value for I^{131} human serum albumin (counted dry) was 41.2 ml. per kilogram with a standard deviation of 5.5 ml. and that for Evans blue dye 40.7 ml. per kilogram with a standard deviation of 4.4 ml.

Wet counting of samples gave a slightly smaller value, 39.7 ml. per kilogram, standard deviation 5.3 ml., than the dry-counting method. Counting whole blood with a scintillation counter produced a value of 73.3 ml. with a standard deviation of 8.8 ml. compared to 71.4 ml. with a standard deviation of 7.3 ml. when hematocrit and Evans blue dye plasma volumes were used.

Values for lean body mass done by antipyrine and specific gravity methods showed an average deviation of 3 kilograms.

The smallest standard deviation from the line of regression occurred when plasma volume was plotted against weight or surface area. It appears from this study, that these two denominators offer the best method for evaluating or predicting plasma volume. The slope for the line of regression for Evans blue dye related to surface area was: plasma volume = 1,770 ml. × surface area M_2 − 460 ml. The value for body weight was: plasma volume = 668 ml. + 30 ml. × weight in kilograms.

We wish to express our gratitude to Drs. J. M. Hayman, Jr., William D. Holden, and Walter H. Pritchard for their helpful suggestions and to Dr. A. S. Littell for statistical analysis of the data.

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THE DESOXYRIBOSE NUCLEIC ACID (DNA) CONTENT IN SPERMATOZOA OF REPEATED SEMINAL FLUIDS FROM FERTILE AND INFERTILE MEN

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IN 1953, Leuchtenberger, Schrader, Weir, and Gentile[1] published the first microspectrophotometric data on the amount of DNA in individual human spermatozoa. They presented evidence that the amount of DNA in the spermatozoa of 21 fertile men examined was very constant and uniform within each individual and among 21 different individuals. The mean DNA content for the spermatozoa of fertile men was given as 1.22* with a standard error of ±0.005, a DNA value which corresponds to approximately one-half of that contained in diploid somatic human cells.[2] In contrast to this characteristic DNA value for the spermatozoa of fertile men, some of the male partners in barren marriages showed significantly lower DNA values in their spermatozoa, although the spermatozoa had a normal morphologic appearance.

Since these data suggested a correlation between a deficiency of DNA in the spermatozoa and one type of male sterility, the possibility of using the DNA content of spermatozoa as a tool for gauging male infertility seemed worth while considering. However, before doing so, further data were needed, especially data which would permit an answer to some of the pertinent questions which are investigated in the following report.

How characteristic is the DNA value in the spermatozoa for an individual over a period of time? In other words, do the spermatozoa of seminal fluids taken at different intervals show the same normal DNA value for the same man with suspected infertility? In an attempt to answer this question the DNA content was determined microspectrophotometrically in nearly 3,000 individual sperms with a normal cytologic appearance in a group of men with proved fertility and in a group of men with suspected infertility. It is to be emphasized that in both the fertile and the infertile groups only morphologically normal sperms were measured. Abnormal forms were excluded.

In this study evidence is presented that the DNA content of spermatozoa obtained at different periods from the fertile men is constant for each individual. By contrast, the DNA content of spermatozoa from men with suspected infertility shows marked fluctuations in individual cases.

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*Arbitrary units.

MATERIAL AND METHODS

In order to carry out the investigation to be described, seminal fluids of both fertile men and men with suspected infertility were secured. The fertile men represented 13 medical students who are married and each of whom is the father of 1 to 3 children. The 21 men with suspected infertility came from the Infertility Clinic of the Maternal Health Association of Cleveland and all were husbands in childless marriages.

The clinical studies of the infertile couples were performed according to the standards established by the American Society for the Study of Sterility[3] except for the fact that endometrial biopsies were not done. Ovulation was assumed to occur if the basal body temperature curves were normal. Additional procedures such as salpingograms, assays of hormone excretion, and psychiatric studies were done when indicated. These clinical studies were the basis for the selection of the men designated as being suspected of infertility. None of the wives of these men showed significant defects which could be presumed to account for the barren marriage, although, of course, this by no means ruled out the possibility that the wife was the infertile member of the couple. The 21 infertile couples were selected from a group of 250 within our total series of 550 couples. Each man of both groups brought in from 5 to 12 specimens of seminal fluid obtained at intervals varying from 1 month to 25 months.

The amount of DNA in the individual spermatozoa was determined microspectrophotometrically as previously described.[1] Microspectrophotometry of the Feulgen stain for the estimation of DNA has been extensively used in recent years on a great variety of materials and by a large number of investigators (for review, see Pollister,[4] Swift,[5] Davidson,[6] and Leuchtenberger[7]). The validity of the method for DNA determinations in spermatozoa as well as in nuclei of other cells has been established by comparing it with other standard methods, such as chromosomal counts, biochemical analysis, and ultraviolet microspectrophotometry.[8]

In this study, the amounts of DNA in spermatozoa are expressed, as in our previous studies,[1] in the same arbitrary units which are used by other workers in the field of microspectrophotometry. A factor of 2×10^{-9} mg. will convert these arbitrary units to absolute amounts of DNA.[2]

RESULTS

In Fig. 1 the distributions of the mean amounts of DNA per spermatozoon for each of 55 specimens from men with proved fertility and for each of 118 specimens from 21 men with suspected infertility are presented. For the 13 men with proved fertility, specimens were taken at 1- to 25-month intervals; for the 21 men with suspected infertility, the specimens were taken at 1- to 15-month intervals.

It is evident that the mean DNA content of all the samples of the fertile men fall within the narrow range of 1.1-1.4, while the repeat samples of the men with suspected infertility show a much larger scatter in their DNA content, falling in the range of 0.3-1.7.

A further illustration for these diverging results of DNA content in spermatozoa of fertile men and men with suspected infertility is given in Fig. 2, where the DNA measurements of 968 individual spermatozoa of 13 fertile men are compared with 1,891 individual spermatozoa of 21 men with suspected infertility. Ninety-three per cent of all the 968 spermatozoa from fertile men contained a DNA value within the range of 1.05-1.35, while only 39 per cent of the sperma-

tozoa of the men with suspected infertility fell within this range. Again, most of the spermatozoa have a DNA value significantly lower than that of the fertile men.

On the basis of these data, it appears to be established that the mean DNA value of 1.23 ± 0.005 in spermatozoa of fertile men remains constant in repeated samples collected at intervals of 1 to 25 months. This finding is all the more significant, since it is in full accordance with data previously reported.[1]

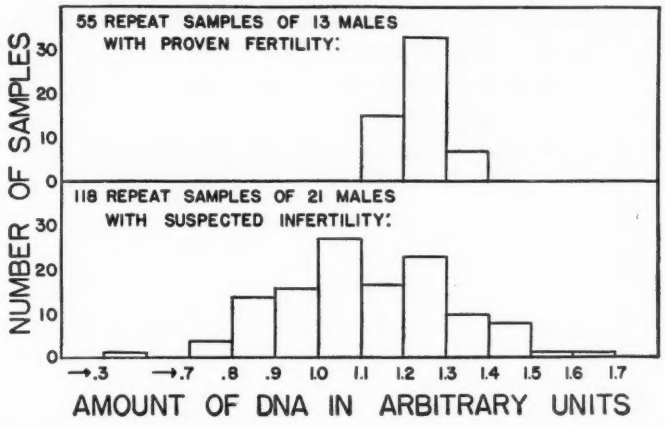

Fig. 1.—Mean amount of DNA in 968 spermatozoa of 13 men with proved fertility (55 repeat samples) and in 1,891 spermatozoa of 21 men with suspected infertility (118 repeat samples).

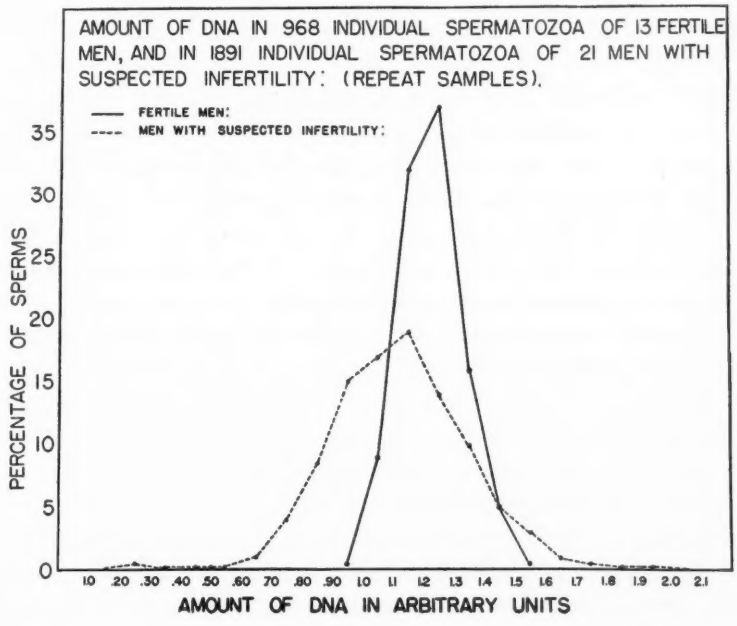

Fig. 2.

On the other hand, samples of spermatozoa derived from the group with suspected infertility do not show such a constancy, but show fluctuations from low, to normal, to high values of DNA. Since the data described so far are group data, an individual case chosen at random from each group is presented in Table I and Fig. 3.

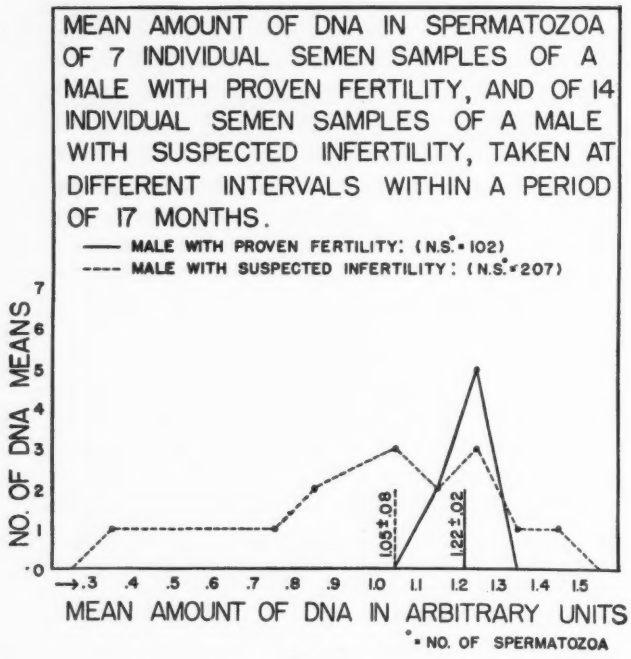
MEAN AMOUNT OF DNA IN SPERMATOZOA OF 7 INDIVIDUAL SEMEN SAMPLES OF A MALE WITH PROVEN FERTILITY, AND OF 14 INDIVIDUAL SEMEN SAMPLES OF A MALE WITH SUSPECTED INFERTILITY, TAKEN AT DIFFERENT INTERVALS WITHIN A PERIOD OF 17 MONTHS.
—— MALE WITH PROVEN FERTILITY: (N.S.°=102)
---- MALE WITH SUSPECTED INFERTILITY: (N.S.°=207)
NO. OF DNA MEANS
7
6
5
4
3
2
1
·0
1.05±.08
1.22±.02
→.3 .4 .5 .6 .7 .8 .9 1.0 1.1 1.2 1.3 1.4 1.5
MEAN AMOUNT OF DNA IN ARBITRARY UNITS
°= NO. OF SPERMATOZOA

Fig. 3.

In Table I and Fig. 3, the mean amounts of DNA for the spermatozoa of a man with proved fertility are compared with those of a man with suspected infertility. It is evident that the DNA content in the spermatozoa of the fertile man in seven seminal samples taken during a period of 1 to 13 months stays remarkably constant. Five of the seven specimens show a value of 1.2 and 1.3; and two, between 1.1-1.2. In other words, all are within the normal range and the largest deviation from the mean is less than 10 per cent. This constancy is in marked contrast to the mean DNA values in the spermatozoa in 14 seminal fluids obtained from the man with suspected infertility. The same man shows a mean DNA value as low as 0.3 in one sample, and as high as 1.43 in the spermatozoa of another sample. In six samples, the mean DNA content of the spermatozoa of this man with suspected infertility fell even within the normal range, a finding the significance of which will be discussed later.

The divergency between the constancy of DNA content in repeat samples from fertile men and the fluctuating DNA values in repeat samples from infertile men is even more apparent if the DNA data of the *individual* sperms in repeat samples from both groups are compared. In Figs. 4 and 5, the individual DNA

TABLE I. MEAN AMOUNTS OF DNA IN SPERMATOZOA OF SEMINAL FLUIDS OBTAINED AT DIFFERENT INTERVALS FROM A FERTILE MAN AND FROM A MAN WITH SUSPECTED INFERTILITY

FERTILE MAN			MAN WITH SUSPECTED INFERTILITY		
SEMINAL FLUIDS	DATE OBTAINED	MEAN AMOUNT OF DNA	SEMINAL FLUIDS	DATE OBTAINED	MEAN AMOUNT OF DNA
1st	12/16/52	1.27 ± 0.00	1st	12/20/51	0.87 ± 0.02
2nd	1/13/53	1.21 ± 0.02	2nd	1/28/52	0.32 ± 0.02
3rd	2/12/53	1.28 ± 0.02	3rd	5/15/52	1.35 ± 0.03
4th	10/ 7/53	1.15 ± 0.02	4th	6/24/52	1.01 ± 0.03
5th	11/11/53	1.24 ± 0.01	5th	7/23/52	0.89 ± 0.03
6th	12/17/53	1.16 ± 0.01	6th	9/ 4/52	1.08 ± 0.02
7th	1/20/54	1.27 ± 0.04	7th	11/13/52	1.10 ± 0.02
			8th	12/29/52	1.13 ± 0.02
			9th	1/29/53	1.26 ± 0.03
			10th	2/26/53	1.29 ± 0.04
			11th	3/12/53	1.43 ± 0.04
			12th	4/ 8/53	0.72 ± 0.01
			13th	4/22/53	1.21 ± 0.03
			14th	5/20/53	1.05 ± 0.02

data for the spermatozoa of the same fertile man and the same infertile man, for whom data are given in Fig. 3 and Table I, are graphed. Besides the dates at which the seminal fluids were obtained, the figures also contain the sperm count by routine analysis of each seminal fluid expressed in millions (M) per cubic centimeter, as well as the sperm motility expressed in per cent. There is also a note on the type of therapy to which the individual had been subjected. The FE numbers are specimen numbers.

It is evident from Fig. 4 that there is not only a marked agreement between the mean DNA contents but there is also a striking similarity between the curves of the *individual* DNA values in spermatozoa of seminal fluids obtained at different periods from the fertile man. It appears that the DNA content of normal spermatozoa of fertile men does indeed stay constant over the time period tested. It is interesting to note the very low sperm counts of this fertile man: 2, 4, 5, 6, 6, 8, 32 millions per cubic centimeter.

The picture is completely different if we examine Fig. 5, in which the individual DNA data for the spermatozoa of the man with suspected infertility are graphed. Here one finds not only a marked variation in the mean DNA contents of the same man within a short time period, but the distribution of the DNA data within a group of spermatozoa varies also from sample to sample. There are curves which resemble the normal distribution curves, while others have either a skewed appearance, two well-separated peaks, or show a rather large scatter of the DNA values.

It can be noted that this irregular pattern of the DNA content in the spermatozoa of the group with suspected infertility is not changed significantly during the period of thyroid therapy to which some of these men had been subjected.*

Similar fluctuations of the DNA values in spermatozoa of repeat samples were also observed in the remaining 19 infertile men while repeat samples from

*A detailed report of the effect of thyroid treatment will be published elsewhere.

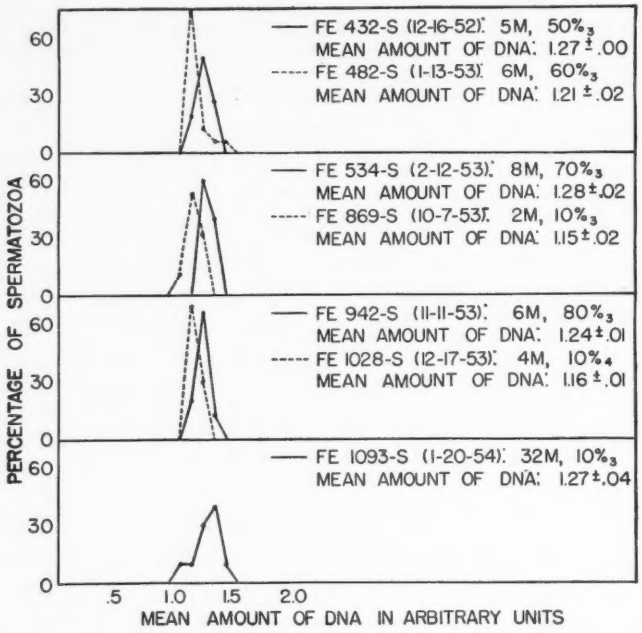
FE 432-S (12-16-52): 5M, 50%
MEAN AMOUNT OF DNA: 1.27 ± .00
FE 482-S (1-13-53): 6M, 60%
MEAN AMOUNT OF DNA: 1.21 ± .02
FE 534-S (2-12-53): 8M, 70%
MEAN AMOUNT OF DNA: 1.28 ± .02
FE 869-S (10-7-53): 2M, 10%
MEAN AMOUNT OF DNA: 1.15 ± .02
FE 942-S (11-11-53): 6M, 80%
MEAN AMOUNT OF DNA: 1.24 ± .01
FE 1028-S (12-17-53): 4M, 10%
MEAN AMOUNT OF DNA: 1.16 ± .01
FE 1093-S (1-20-54): 32M, 10%
MEAN AMOUNT OF DNA: 1.27 ± .04
PERCENTAGE OF SPERMATOZOA
MEAN AMOUNT OF DNA IN ARBITRARY UNITS

Fig. 4.—Amount of DNA (microspectrophotometry) in the spermatozoa of the same fertile man at different intervals.

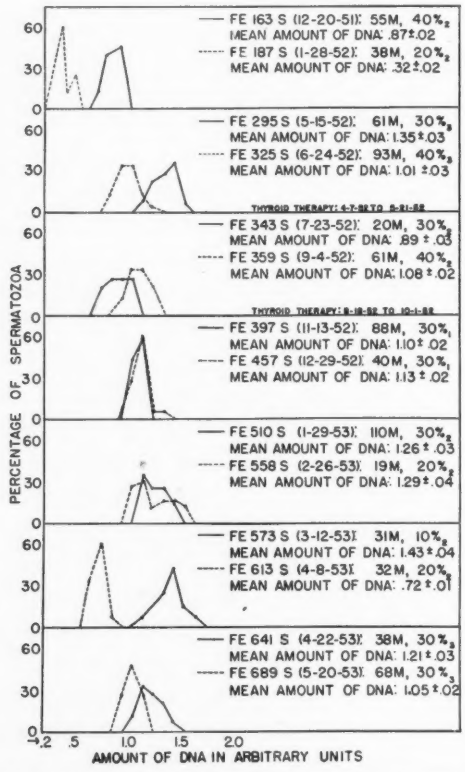
FE 163 S (12-20-51): 55M, 40%
MEAN AMOUNT OF DNA: .87 ± .02
FE 187 S (1-28-52): 38M, 20%
MEAN AMOUNT OF DNA: .32 ± .02
FE 295 S (5-15-52): 61M, 30%
MEAN AMOUNT OF DNA: 1.35 ± .03
FE 325 S (6-24-52): 93M, 40%
MEAN AMOUNT OF DNA: 1.01 ± .03
THYROID THERAPY: 4-7-52 TO 5-21-52
FE 343 S (7-23-52): 20M, 30%
MEAN AMOUNT OF DNA: .89 ± .03
FE 359 S (9-4-52): 61M, 40%
MEAN AMOUNT OF DNA: 1.08 ± .02
THYROID THERAPY: 8-18-52 TO 10-1-52
FE 397 S (11-13-52): 88M, 30%
MEAN AMOUNT OF DNA: 1.10 ± .02
FE 457 S (12-29-52): 40M, 30%
MEAN AMOUNT OF DNA: 1.13 ± .02
FE 510 S (1-29-53): 110M, 30%
MEAN AMOUNT OF DNA: 1.26 ± .03
FE 558 S (2-26-53): 19M, 20%
MEAN AMOUNT OF DNA: 1.29 ± .04
FE 573 S (3-12-53): 31M, 10%
MEAN AMOUNT OF DNA: 1.43 ± .04
FE 613 S (4-8-53): 32M, 20%
MEAN AMOUNT OF DNA: .72 ± .01
FE 641 S (4-22-53): 38M, 30%
MEAN AMOUNT OF DNA: 1.21 ± .03
FE 689 S (5-20-53): 68M, 30%
MEAN AMOUNT OF DNA: 1.05 ± .02
PERCENTAGE OF SPERMATOZOA
AMOUNT OF DNA IN ARBITRARY UNITS

Fig. 5.—Amount of DNA (microspectrophotometry) in spermatozoa of the same man with suspected infertility taken at different intervals.

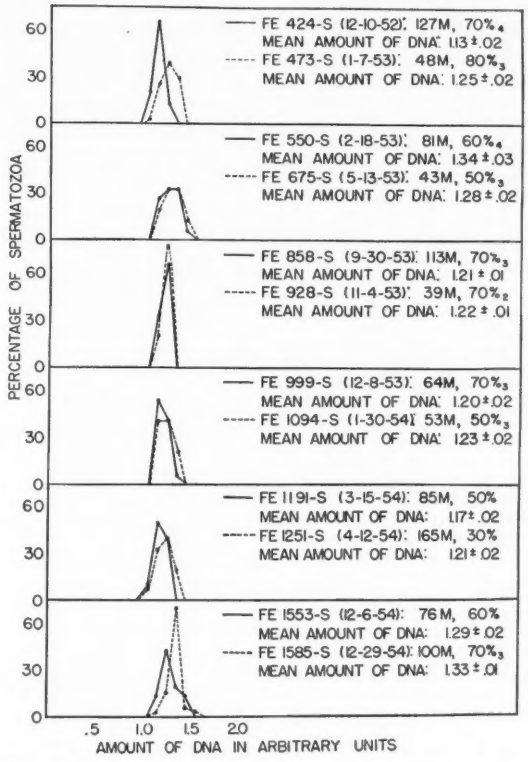
FE 424-S (12-10-52): 127M, 70%4
MEAN AMOUNT OF DNA: 1.13±.02
FE 473-S (1-7-53): 48M, 80%3
MEAN AMOUNT OF DNA: 1.25±.02
FE 550-S (2-18-53): 81M, 60%4
MEAN AMOUNT OF DNA: 1.34±.03
FE 675-S (5-13-53): 43M, 50%3
MEAN AMOUNT OF DNA: 1.28±.02
FE 858-S (9-30-53): 113M, 70%3
MEAN AMOUNT OF DNA: 1.21±.01
FE 928-S (11-4-53): 39M, 70%2
MEAN AMOUNT OF DNA: 1.22±.01
FE 999-S (12-8-53): 64M, 70%3
MEAN AMOUNT OF DNA: 1.20±.02
FE 1094-S (1-30-541 53M, 50%3
MEAN AMOUNT OF DNA: 1.23±.02
FE 1191-S (3-15-54): 85M, 50%
MEAN AMOUNT OF DNA: 1.17±.02
FE 1251-S (4-12-54): 165M, 30%
MEAN AMOUNT OF DNA: 1.21±.02
FE 1553-S (12-6-54): 76M, 60%
MEAN AMOUNT OF DNA: 1.29±.02
FE 1585-S (12-29-54): 100M, 70%3
MEAN AMOUNT OF DNA: 1.33±.01
PERCENTAGE OF SPERMATOZOA
60
30
0
.5
1.0
1.5
2.0
AMOUNT OF DNA IN ARBITRARY UNITS

Fig. 6.—Amount of DNA (microspectrophotometry) in the spermatozoa of the same fertile man at different intervals.

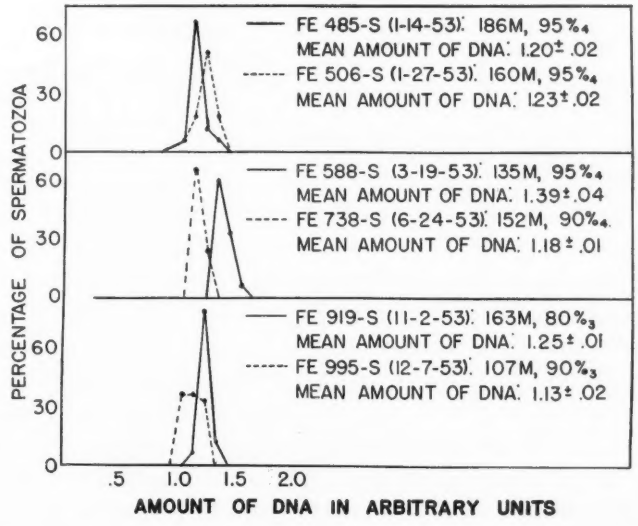

Fig. 7.—Amount of DNA (microspectrophotometry) in the spermatozoa of the same fertile man at different intervals.

the remaining 11 fertile men showed again the same striking constancy of the DNA content for the spermatozoa. A few representative examples are given in Figs. 6 to 10.

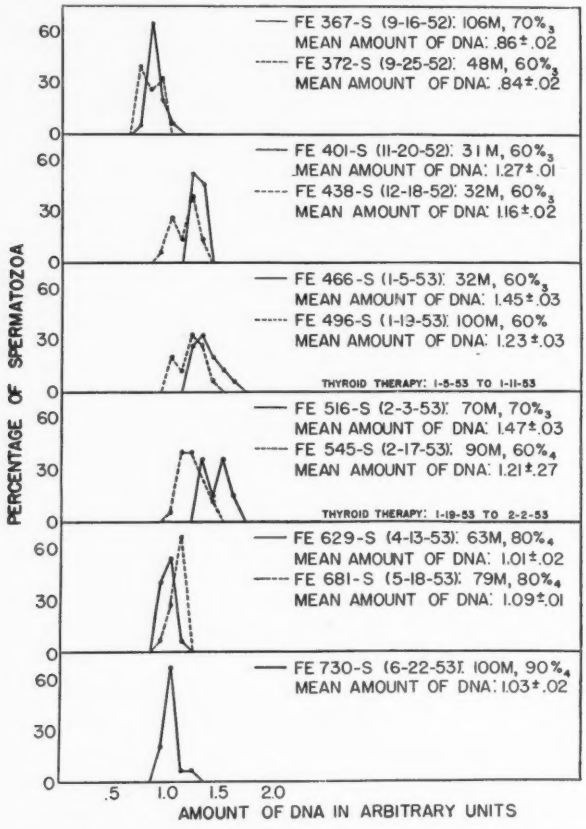
FE 367-S (9-16-52): 106M, 70%
MEAN AMOUNT OF DNA: .86±.02
FE 372-S (9-25-52): 48M, 60%
MEAN AMOUNT OF DNA: .84±.02
FE 401-S (11-20-52): 31M, 60%
MEAN AMOUNT OF DNA: 1.27±.01
FE 438-S (12-18-52): 32M, 60%
MEAN AMOUNT OF DNA: 1.16±.02
FE 466-S (1-5-53): 32M, 60%
MEAN AMOUNT OF DNA: 1.45±.03
FE 496-S (1-13-53): 100M, 60%
MEAN AMOUNT OF DNA: 1.23±.03
THYROID THERAPY: 1-5-53 TO 1-11-53
FE 516-S (2-3-53): 70M, 70%
MEAN AMOUNT OF DNA: 1.47±.03
FE 545-S (2-17-53): 90M, 60%
MEAN AMOUNT OF DNA: 1.21±.27
THYROID THERAPY: 1-19-53 TO 2-2-53
FE 629-S (4-13-53): 63M, 80%
MEAN AMOUNT OF DNA: 1.01±.02
FE 681-S (5-18-53): 79M, 80%
MEAN AMOUNT OF DNA: 1.09±.01
FE 730-S (6-22-53): 100M, 90%
MEAN AMOUNT OF DNA: 1.03±.02
PERCENTAGE OF SPERMATOZOA
60
30
0
.5
1.0
1.5
2.0
AMOUNT OF DNA IN ARBITRARY UNITS

Fig. 8.—Amount of DNA (microspectrophotometry) in spermatozoa of the same man with suspected infertility taken at different intervals.

DISCUSSION

The results of the DNA measurements in repeated seminal samples of spermatozoa of fertile men and men with suspected infertility may be summarized as follows:

1. Fertile men show a remarkably constant DNA content in their spermatozoa in all seminal fluids obtained within a period of over two years.

2. Men who are partners in childless marriages and who are designated in this study as men with suspected infertility do not show a constant DNA value in their spermatozoa, although these spermatozoa were of normal morphologic appearance. The DNA content varies in spermatozoa of repeated seminal samples from low, to normal, to high values; in other words, reveals a whole range of variations.

1. *Constancy of the DNA Content in Spermatozoa of Fertile Men.*—Considering first the constancy of the DNA content in spermatozoa of fertile men,

the results in this study are in full agreement with those of the previous investigation by Leuchtenberger, Schrader, Weir, and Gentile.[1] The similarity of the DNA data is indeed well demonstrated if the graph of the previous study, representing data for 563 spermatozoa, is compared with the present one, which represents the DNA data for 968 spermatozoa, Fig. 11.

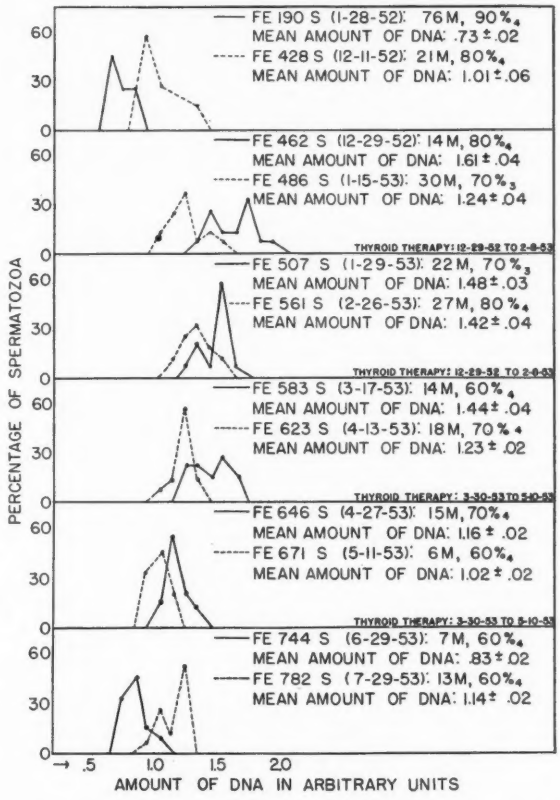
FE 190 S (1-28-52): 76M, 90%
MEAN AMOUNT OF DNA: .73 ± .02
FE 428 S (12-11-52): 21M, 80%
MEAN AMOUNT OF DNA: 1.01 ± .06
FE 462 S (12-29-52): 14M, 80%
MEAN AMOUNT OF DNA: 1.61 ± .04
FE 486 S (1-15-53): 30M, 70%
MEAN AMOUNT OF DNA: 1.24 ± .04
THYROID THERAPY: 12-29-52 TO 2-8-53
FE 507 S (1-29-53): 22M, 70%
MEAN AMOUNT OF DNA: 1.48 ± .03
FE 561 S (2-26-53): 27M, 80%
MEAN AMOUNT OF DNA: 1.42 ± .04
THYROID THERAPY: 12-29-52 TO 2-8-53
FE 583 S (3-17-53): 14M, 60%
MEAN AMOUNT OF DNA: 1.44 ± .04
FE 623 S (4-13-53): 18M, 70%
MEAN AMOUNT OF DNA: 1.23 ± .02
THYROID THERAPY: 3-30-53 TO 5-10-53
FE 646 S (4-27-53): 15M, 70%
MEAN AMOUNT OF DNA: 1.16 ± .02
FE 671 S (5-11-53): 6M, 60%
MEAN AMOUNT OF DNA: 1.02 ± .02
THYROID THERAPY: 3-30-53 TO 5-10-53
FE 744 S (6-29-53): 7M, 60%
MEAN AMOUNT OF DNA: .83 ± .02
FE 782 S (7-29-53): 13M, 60%
MEAN AMOUNT OF DNA: 1.14 ± .02
PERCENTAGE OF SPERMATOZOA
60
30
0
.5
1.0
1.5
2.0
AMOUNT OF DNA IN ARBITRARY UNITS

Fig. 9.—Amount of DNA (microspectrophotometry) in spermatozoa of the same man with suspected infertility taken at different intervals.

It can be seen that the mean DNA values of both studies are practically identical, namely, 1.22 ± 0.005 and 1.23 ± 0.005 for 21 and 13 men, respectively. Furthermore, the type of curve of the previous study is practically identical with the present one, indicating the same narrow scatter of the DNA content within the spermatozoa.

Since both combined studies represent the results of DNA measurements in nearly 1,400 individual spermatozoa of the fertile group, this constancy of the DNA content in spermatozoa of fertile men can be considered as fully established. This finding, however, applies only to the spermatozoa which are cytologically of normal appearance. The possibility that the DNA content in the abnormal spermatozoa (which are also present in different numbers in each seminal fluid of fertile men) is different cannot be excluded. Indeed the chemical

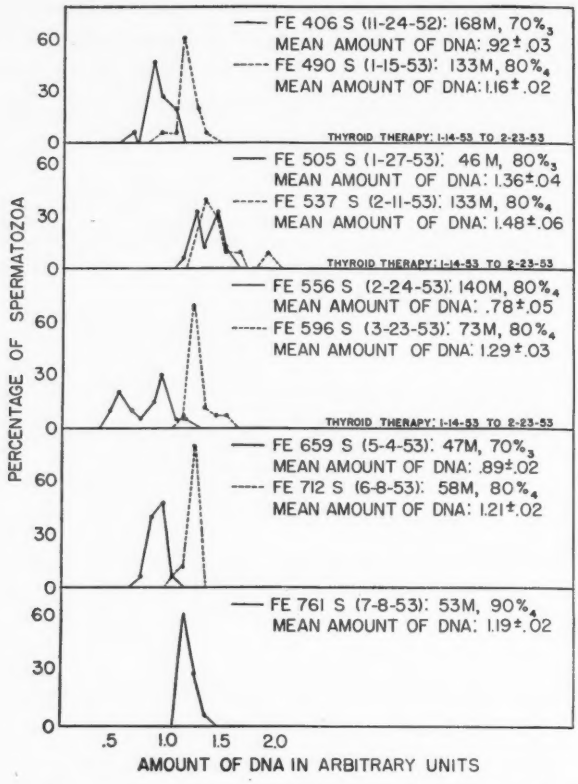
FE 406 S (11-24-52): 168M, 70%3
MEAN AMOUNT OF DNA: .92±.03
FE 490 S (1-15-53): 133M, 80%4
MEAN AMOUNT OF DNA: 1.16±.02
THYROID THERAPY: 1-14-53 TO 2-23-53
FE 505 S (1-27-53): 46 M, 80%3
MEAN AMOUNT OF DNA: 1.36±.04
FE 537 S (2-11-53): 133M, 80%4
MEAN AMOUNT OF DNA: 1.48±.06
THYROID THERAPY: 1-14-53 TO 2-23-53
FE 556 S (2-24-53): 140M, 80%4
MEAN AMOUNT OF DNA: .78±.05
FE 596 S (3-23-53): 73M, 80%4
MEAN AMOUNT OF DNA: 1.29±.03
THYROID THERAPY: 1-14-53 TO 2-23-53
FE 659 S (5-4-53): 47M, 70%3
MEAN AMOUNT OF DNA: .89±.02
FE 712 S (6-8-53): 58M, 80%4
MEAN AMOUNT OF DNA: 1.21±.02
FE 761 S (7-8-53): 53M, 90%4
MEAN AMOUNT OF DNA: 1.19±.02
PERCENTAGE OF SPERMATOZOA
60
30
0
.5
1.0
1.5
2.0
AMOUNT OF DNA IN ARBITRARY UNITS

Fig. 10.—Amount of DNA (microspectrophotometry) in spermatozoa of the same man with suspected infertility taken at different intervals.

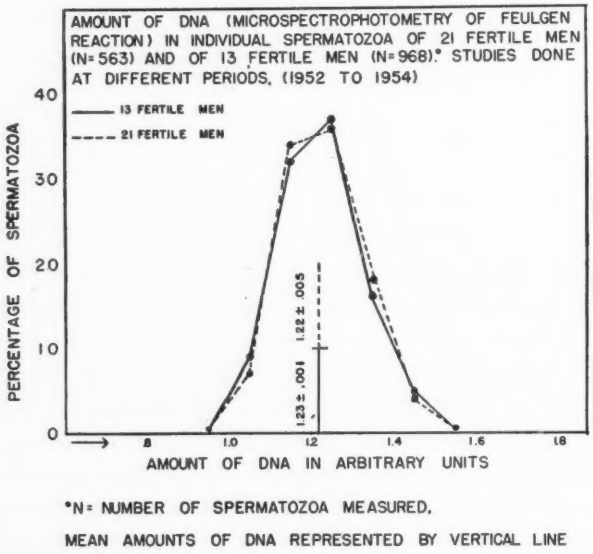

Fig. 11.

determinations on sperm suspensions favor such a concept. Davidson, Leslie, and White[9] determined the DNA content in "11 samples of semen which had been found normal." They found within this group a range from 1.8-5.8 $\times$ 10^{-7} mg. per sperm. While such a wide range of DNA data for spermatozoa of fertile men seems indeed surprising, the divergency of these results from the ones reported previously[1] and in the present study may be explained if one considers the data in the light of the method employed.

The biochemical analysis for DNA is done on a *suspension* of a known number of spermatozoa, and the final DNA value for a sperm is computed by dividing the total DNA value by the number of spermatozoa analyzed. As pointed out repeatedly,[2, 7, 10] unless each of the sperms in such a suspension contains the same amount of DNA (which in view of the admixture of varying numbers of abnormal sperms is indeed out of the question), the computed DNA value is by no means representative for the morphologically normal sperms which were individually examined in the microspectrophotometric studies.

A consideration of this thought may perhaps explain the discordant results which 3 groups of biochemical workers[11-13] have obtained on analyzing the DNA content of sperm suspensions from the bull. Since, from our own studies,[7, 14, 15] we know that in seminal fluids of bulls there are also varying numbers of morphologically abnormal spermatozoa besides the normal ones, different ratios between normal and abnormal spermatozoa may explain the different DNA values for each of the biochemical analyses.

In view of the microspectrophotometric results, which consistently showed a constant DNA value for morphologically normal sperms of fertile men, the wide range obtained by chemical studies on sperm suspensions of fertile men by Davidson and associates[9] is probably due to the masking of the DNA value of these normal sperms by those of abnormal sperms and other cellular elements so frequently found in seminal fluids.* Actually one of the great advantages of the microspectrophotometric method is the possibility of analyzing *single cells under the microscope.* Thus this method permits the selection of only morphologically normal sperms for the DNA analysis.

2. *Variability of the DNA Content in Spermatozoa of Men With Suspected Infertility.*—In discussing the fluctuations, it should be stressed again that these results were obtained only on spermatozoa which were selected for measuring because of their normal morphology. That is, each sperm measured in the group with suspected infertility was cytologically indistinguishable from the sperms measured in the fertile group. This statement is supported by the data in Fig. 12, in which the nuclear diameters of 912 individual spermatozoa from fertile men are compared with the nuclear diameters of 1,772 individual spermatozoa from infertile men. It can be seen that in spite of the divergent DNA values obtained in the fertile and infertile groups (Figs. 1 and 2) the mean nuclear diameters indeed are identical for both the fertile and the infertile groups. Therefore, it seems that the difference between the DNA content in spermatozoa

*Davidson agrees with this interpretation (personal communication).

of fertile men and men with suspected infertility is all the more significant since it reveals a disturbance which cannot be detected by a morphologic study alone.

The fluctuations in the DNA content of cytologically normal spermatozoa obtained at different periods from the same man with suspected infertility as contrasted with the constant DNA content in the fertile man is a finding which deserves special consideration. One may say that in view of the extreme constancy in spermatozoa of fertile men, one may have suspected that a deviation in the DNA content (either too high or too low) in the man with suspected infertility may also be a persistent feature. But the results in this study are certainly in complete contradiction to such a concept.

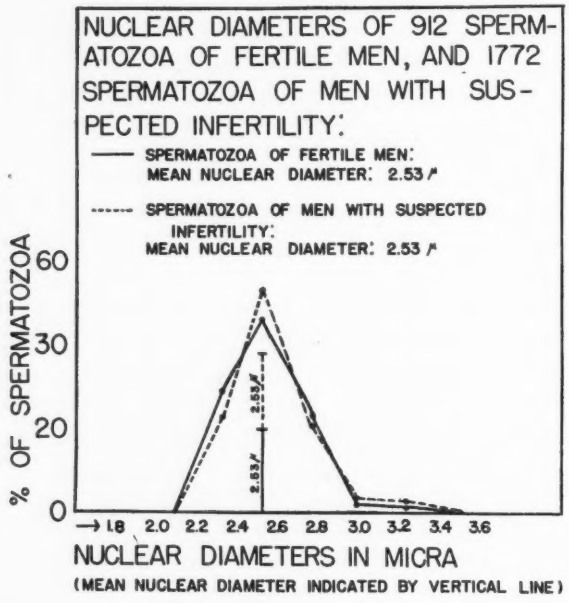
NUCLEAR DIAMETERS OF 912 SPERMATOZOA OF FERTILE MEN, AND 1772 SPERMATOZOA OF MEN WITH SUSPECTED INFERTILITY.
SPERMATOZOA OF FERTILE MEN:
MEAN NUCLEAR DIAMETER: 2.53 μ
SPERMATOZOA OF MEN WITH SUSPECTED INFERTILITY:
MEAN NUCLEAR DIAMETER: 2.53 μ
% OF SPERMATOZOA
60
30
20
0
2.53 μ
2.53 μ
1.8 2.0 2.2 2.4 2.6 2.8 3.0 3.2 3.4 3.6
NUCLEAR DIAMETERS IN MICRA
(MEAN NUCLEAR DIAMETER INDICATED BY VERTICAL LINE)

Fig. 12.

Neither a too low nor a too high DNA value in the spermatozoa is of necessity a persistent feature but may change back to the normal value. While this normal DNA value in infertile men may again return to a faulty DNA value, nevertheless the fact exists that the disturbance is not necessarily irreversible for each individual. Further studies may perhaps reveal 3 groups: the fertile men with the constant normal DNA values; the men with suspected infertility with fluctuations in their DNA values from low to normal to high or vice versa; and the men with suspected infertility with a persistently too high or too low value.

But at the present time, the fact that a man with deviating DNA values may at some time have a normal DNA content in his spermatozoa must be taken into consideration, especially when trying to evaluate the DNA data in spermatozoa. A DNA analysis from a single seminal sample which gives a normal

DNA value is by no means indicative that the individual belongs in the fertile group. Only DNA examinations of repeated samples at various time intervals will reveal the DNA pattern and determine in which group the individual belongs.

Although it is conceivable that a man with an occasional normal DNA value might be fertile at this period, this is by no means assured, but needs further study. Perhaps the frequency with which the normal DNA values recur may be an important factor in gauging the fertility of the sperms.

While it is realized that many more data are needed before the DNA content of cytologically normal spermatozoa can be considered as an established criterion for gauging male infertility, the results in the previous and the present study nevertheless favor the correlation between the disturbance in the DNA content of the sperms and at least one type of male infertility. In further support of this concept are the findings that spermatogenic cells in the testis, such as primary and secondary spermatocytes as well as spermatids from infertile men, contain significantly lower amounts of DNA than do those from fertile men.[16]

SUMMARY

Microspectrophotometric determinations were made on the amounts of DNA in nearly 3,000 individual human spermatozoa of normal cytologic appearance, obtained from 173 seminal samples of 13 fertile men and 21 men with suspected infertility. The results were as follows:

1. The amount of DNA per spermatozoon of the 13 fertile men is very constant and uniform within each individual in all the seminal fluids obtained at intervals from 1 to 25 months and among the 13 different individuals. The mean amount of DNA in arbitrary units is 1.23 ± 0.005 per sperm and is in agreement with the previously reported amount of 1.22 ± 0.005.

2. In contrast to the uniformity of the DNA content in the sperms of fertile men, the sperms from 21 men with suspected infertility showed greatly varying amounts of DNA within each individual in the seminal fluids obtained at intervals from 1 to 15 months and among the 21 different individuals.

3. These data offer further support for the concept that one type of male sterility may be associated with a deviating DNA content in the sperms.

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INFLUENCE OF HYPOPHYSECTOMY UPON THE ESTABLISHED HYPERTENSIVE DISEASE INDUCED BY DESOXYCORTICOSTERONE

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IN 1940, Grollman and associates[1] reported that various steroid derivatives, namely, estradiol, testosterone, desoxycorticosterone, progesterone, and diethylstilboestrol, share the property of inducing hypertension in the rat. Subsequently, Darrow and Miller[2] found that repeated injections of desoxycorticosterone acetate (DCA) resulted in the production of cardiac lesions indistinguishable from the ones induced by diets low in potassium. Selye and coworkers[3] have demonstrated, in a series of papers, that the ability to produce hypertension seems to be a specific property of mineralocorticoids, that DCA in animals prepared with unilateral nephrectomy and drinking 1 per cent NaCl readily produces hypertension, nephrosclerosis, myocarditis, periarteritis nodosa, and polyuria, and that the cardiac lesions are not due to hypopotassemia. There seems to exist now general agreement (irrespective of any possible clinical implication) that DCA produces an experimental syndrome reminiscent in many of its features of the hypertensive disease of human beings.

It has been shown that hypophysectomy inhibits the development of the hypertension[4] and the accompanying lesions[5, 6] induced by DCA. It has been shown, too,[5] that hypophysectomy inhibits the hypertension that persists after the discontinuation of a treatment with small doses of DCA (metacorticoid hypertension), but in this work no reference was made to the fate of the pathologic lesions.

We wish to report here some experiments dealing with the effect of hypophysectomy on the established hypertension and pathologic syndrome induced by DCA.

MATERIALS AND METHODS

In all, 240 Sprague-Dawley female albino rats were used.

Experiment No. 1.—Sixty-five rats, weighing 60 to 80 grams, were provisionally divided into two groups; the first 30 animals served as controls (Group A); the second (Group B) were treated with DCA (one 20 mg. pellet implanted every week during the first 3 weeks). After 25 days, on the day that we will call "0," 30 animals from Group A and 30 from Group B (the survivors) were then divided into groups of 10 animals each, as outlined in Table I. The animals were observed for 18 days and then sacrificed.

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TABLE I. ORGAN WEIGHTS AND QUANTITATIVE EVALUATION OF THE PATHOLOGIC LESIONS IN THE ANIMALS OF EXPERIMENT NO. 1

GROUP AND TREATMENT	FINAL NUMBER OF RATS*	ORGAN WEIGHTS IN MG./100 GRAMS OF BODY WEIGHT				NEPHROSCLEROSIS		MYOCARDITIS		PERIARTERITIS NODOSA	
		KIDNEY	HEART	TESTIS	ADRENALS	INCIDENCE (PER CENT)	SEVERITY (PER CENT)	INCIDENCE (PER CENT)	SEVERITY (PER CENT)	INCIDENCE (PER CENT)	SEVERITY (PER CENT)
I											
None. Killed at day 0	10	896 ± 26	320 ± 12	1552 ± 55	21 ± 0.7	0	0	0	0	0	0
II											
None	(10) 10	730 ± 41	328 ± 8	1189 ± 61	19 ± 1.8	0	0	0	0	0	0
III											
Hypophysectomy	(13) 10	538 ± 9	270 ± 7	612 ± 42	11 ± 0.28	0	0	0	0	0	0
IV											
DCA. Killed at day 0	10	1288 ± 23	512 ± 31	1302 ± 98	32 ± 1.8	60	33	80	27	30	10
V											
DCA	(10) 7	1348 ± 104	579 ± 31	1327 ± 146	34 ± 2.1	90	93	80	50	50	50
VI											
DCA + hypophysectomy	(10) 5	686 ± 22	442 ± 22	406 ± 85	16 ± 1.1	50	20	60	7	20	7

*The figures in brackets correspond to the initial number of animals.

TABLE II. ORGANS WEIGHTS AND QUANTITATIVE EVALUATION OF PATHOLOGIC LESIONS IN THE ANIMALS OF EXPERIMENT NO. 2

TABLE II. ORGANS WEIGHTS AND QUANTITATIVE EVALUATION OF PATHOLOGIC LESIONS IN THE ANIMALS OF EXPERIMENT NO. 2

GROUP AND TREATMENT	FINAL NUMBER OF RATS*	ORGAN WEIGHTS IN MG./100 GRAMS OF BODY WEIGHT				NEPHROSCLEROSIS		MYOCARDITIS		PERIARTERITIS NODOSA	
		KIDNEY	HEART	TESTIS	ADRENALS	INCIDENCE (PER CENT)	SEVERITY (PER CENT)	INCIDENCE (PER CENT)	SEVERITY (PER CENT)	INCIDENCE (PER CENT)	SEVERITY (PER CENT)
I											
DCA. Killed at day 0	9	1317 ± 137	406 ± 13	1369 ± 69	23 ± 1.1	77	50	88	60	67	20
II											
DCA	(13) 7	1436 ± 123	414 ± 19	1383 ± 72	18 ± 1.4	100	86	92	67	77	44
III											
DCA + hypophysectomy	(13) 11	761 ± 43	408 ± 22	328 ± 31	7 ± 0.16	54	12	82	40	27	11
IV											
DCA + hypophysectomy + LAP + ACTH	(11) 7	1017 ± 71	346 ± 16	782 ± 59	14 ± 1.1	86	28	43	17	29	10
V											
DCA + controlled fasting	(8) 3	1491	451	1372	21	100	89	100	67	50	25

*The figures in brackets correspond to the initial number of animals.

TABLE III. ORGAN WEIGHTS AND QUANTITATIVE EVALUATION OF THE PATHOLOGIC LESIONS IN EXPERIMENT NO. 3

GROUP AND TREATMENT	FINAL NUMBER OF RATS*	ORGAN WEIGHTS IN MG./100 GRAMS OF BODY WEIGHT			NEPHROSCLEROSIS		MYOCARDITIS		PERIARTERITIS NODOSA	
		KIDNEY	HEART	ADRENALS	INCIDENCE (PER CENT)	SEVERITY (PER CENT)	INCIDENCE (PER CENT)	SEVERITY (PER CENT)	INCIDENCE (PER CENT)	SEVERITY (PER CENT)
I										
None	(10) 10	579 ± 29	322 ± 24	14 ± 0.6	0	0	0	0	0	0
II†										
DCA. Killed on day 0	10				100	70	100	65	77	44
III										
DCA	(15) 4	978 ± 59	544 ± 28	26 ± 2.4	100	95	100	91	100	81
IV										
DCA + hypophysectomy	(18) 7	675 ± 15	485 ± 37	12 ± 1.5	100	33	100	47	83	69
V										
DCA + hypophysectomy + STH	(16) 9	715 ± 21	513 ± 43	10 ± 0.44	85	30	75	60	85	80
VI										
DCA + hypophysectomy + thyroxin	(22) 14	740 ± 22	475 ± 16	11 ± 0.7	100	27	83	43	60	60

*The figures in brackets correspond to the initial number of animals.
†No organ weights were taken in this group.

Experiment No. 2.—Sixty-five rats were treated with DCA (in a similar manner as in the preceding experiment) and after 39 days were subdivided into groups, as outlined in Table II. The treatment with lyophilized anterior pituitary material (LAP) consisted of 10 mg. in the first week, 15 mg. in the second, and 20 mg. thereafter, the dose being per animal per day, injected subcutaneously, suspended in 0.4 ml. of 0.85 per cent of NaCl. ACTH (Acthar Gel, Armour) was given subcutaneously twice a day, the daily dose per animal being 1 mg. in the first week, and 2 mg. thereafter. The daily food intake of the animals of Group V was restricted in such a way as to follow as closely as possible the variations in weight of Group III. After 23 days, the surviving animals were sacrificed.

Experiment No. 3.—One hundred ten rats were used: 10 were controls and 100 were implanted at the time of nephrectomy with three 20 mg. DCA pellets. After 34 days, 10 controls and 91 DCA-treated rats were still alive; these were divided into groups, as outlined in Table III. The animals were observed for 12 days and then sacrificed. Somatotropin (STH, Armour Lot R 285-183) was administered twice a day subcutaneously, dissolved in saline solution. The daily dose was 500 μg per animal per day. Thyroxin (Thyroxinsodium, British Drug Houses) was administered once a day dissolved in water, the daily dose being 5 μg per animal per day.

In the experiments all animals were unilaterally nephrectomized (the right kidney being removed) and given 1 per cent NaCl (as drinking fluid) and Purina Fox Chow ad libitum. Fluid intake was measured daily, body weight and blood pressure weekly. The blood pressure determinations were made by the method of Friedman and Freed[7] under light ether anesthesia. The completeness of hypophysectomy was verified by inspection at autopsy and by such criteria as body, adrenal, and testicular weights, and by routine histologic studies of adrenals and testes. Data obtained from incompletely operated animals are omitted. At the conclusion of each experiment, the rats were killed and the organs were dissected, weighed, and stained with hematoxylin and eosin as well as with PAS.[8] An attempt was made to evaluate the pathologic changes on a semiquantitative basis, using a scale of 0 to +++. The percentage of positives in any one group was designated as "per cent incidence" and the means of the values obtained by grading were expressed as a percentage of the possible maximum, and designated as "per cent severity." Statistical calculations were made according to Snedecor.[9]

RESULTS

In giving an account of our results, we will begin with the particulars of each experiment as regards clinical state, blood pressure, etc., whereas the histologic results will be considered conjointly for the three experiments.

Experiment No. 1.—At the moment of the division into groups (on Day 0), the DCA-treated animals were hypertensive (Fig. 1) and, as shown in Table I, had moderate lesions in kidney, heart, and mesentery.

After hypophysectomy, the blood pressure went down toward normal values, the fluid intake did not change very much, and, as seen in the pathologic evaluation of the results, the lesions in the DCA-treated hypophysectomized animals (Group VI) were even less severe than at the moment of the operation (Group IV). In this group, we had a high mortality in DCA-treated hypophysectomized animals (Group IV) mainly during the first days after the operation.

Experiment No. 2.—The results are similar to those of experiment No. 1 and are represented in Fig. 2 and Table II. It can be seen that since the treatment before the operation was longer, the syndrome induced was even more severe; nevertheless, the results are more striking than in the preceding experiment as it appears that the survival of DCA-treated animals is better

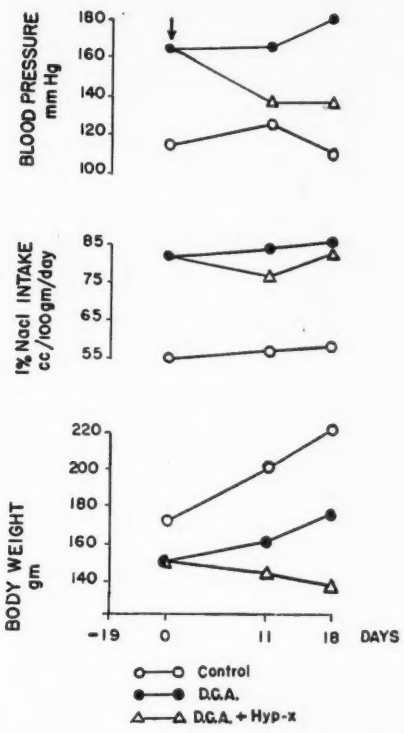


Fig. 1.—Changes in blood pressure, 1 per cent NaCl intake, and body weight of the animals in experiment No. 1 (not including the animals of Group III).

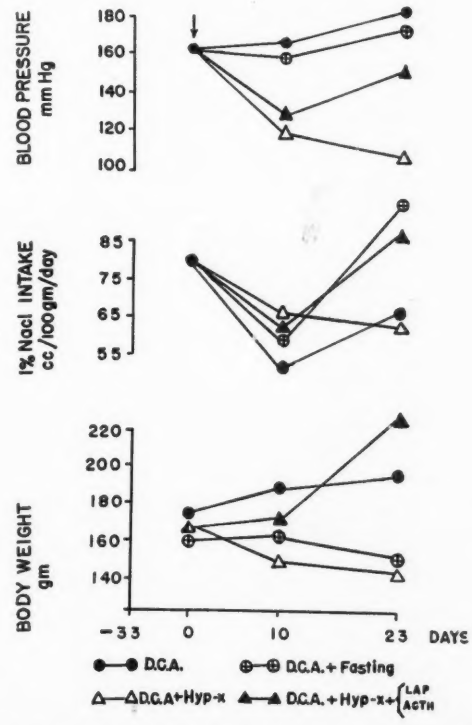
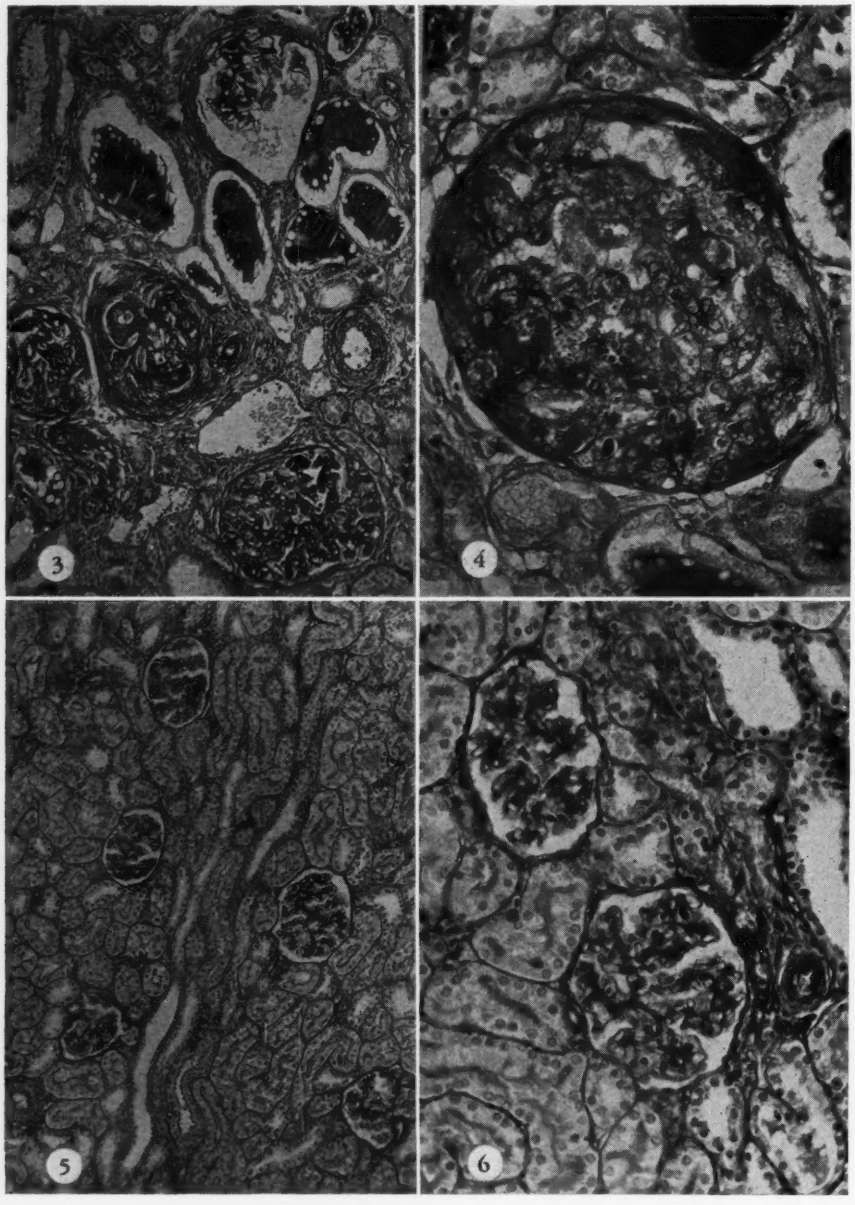


Fig. 2.—Changes in body weight, fluid intake, and blood pressure of the animals in experiment No. 2.

after hypophysectomy. The treatment of hypophysectomized animals with LAP + ACTH induced growth, a higher intake of 1 per cent NaCl than the hypophysectomized animals not treated, and only partially restored the blood pressure to the level attained by intact animals.

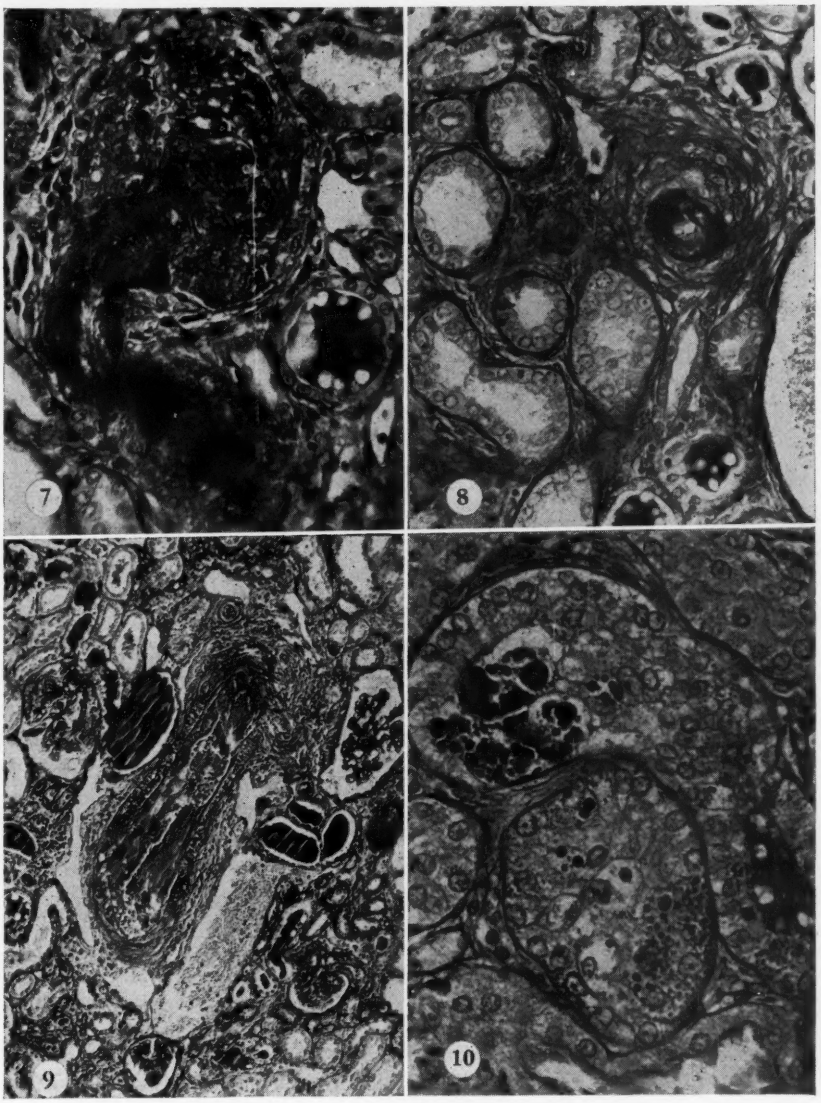


Figs. 3 and 4.—Low- and high-power view of the kidney of a DCA-treated intact rat showing severe lesions (PAS ×120 and ×300, respectively; reduced ¼).

Figs. 5 and 6.—Kidney of a similarly treated animal as in 3 but 12 days after hypophysectomy, showing essentially normal renal structure (PAS ×120 and ×350, respectively; reduced ¼).

The animals on restricted food (Group IV) showed a high blood pressure and, in spite of the small amount of food that they received, a high fluid intake.

Experiment No. 3.—In this experiment, the DCA treatment was particularly toxic for the animals, as evidenced by the high mortality and the incidence and severity of the lesions in Groups I and II; nevertheless, the results are similar to those of the preceding experiments (Table III).



Figs. 7-10.—These figures correspond to the kidneys of DCA-treated intact animals.

Fig. 7.—Hyalinization of afferent arteriole and glomerulus (PAS ×450; reduced ¼).

Fig. 8.—Hyalinization of a renal arteriole. Tubules showing thickening of the basement membranes and absence of brushlike border (PAS ×300; reduced ¼).

Fig. 9.—Interlobar artery with pronounced hyalinization (PAS ×100; reduced ¼).

Fig. 10.—Two tubules with intracellular and intratubular hyaline droplets (PAS ×450; reduced ¼).

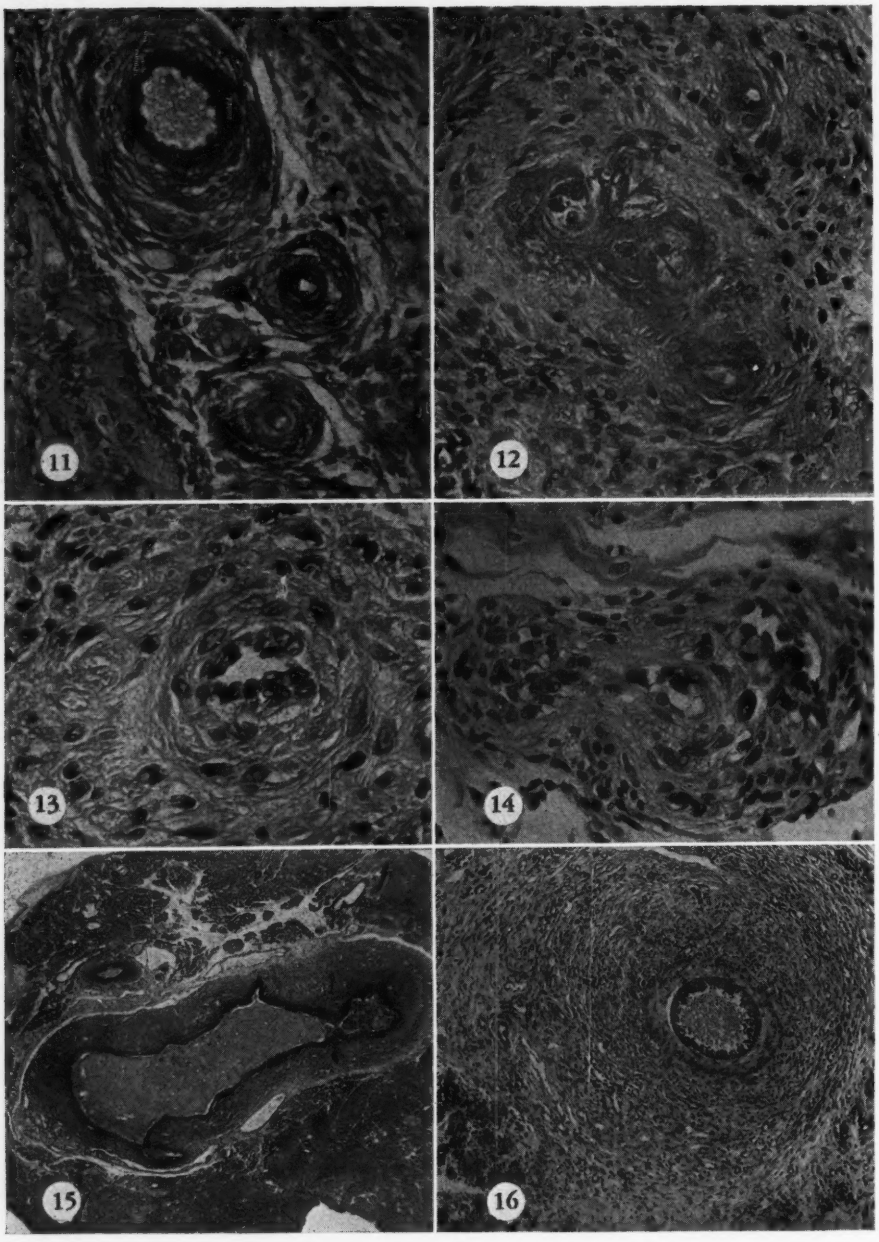


Fig. 11.—Several degress of hyalinization in myocardial arteries of a DCA-treated intact rat (PAS ×300; reduced ⅙).

Fig. 12.—Myocardial nodule in the same animal as in Fig. 11 (hematoxylin and eosin ×300; reduced ⅙).

Figs. 13 and 14.—Myocardial lesions in DCA-treated hypophysectomized animals showing the cellularity of the lesions (hematoxylin and eosin ×540 and ×330, respectively; reduced ⅙).

Fig. 15.—Aneurysm in a pancreatic artery of a DCA-treated intact animal (PAS ×8; reduced ⅙).

Fig. 16.—Periarteritis nodosa in a pancreatic artery of the same animal as in Figs. 5 and 6 (hematoxylin and eosin ×75; reduced ⅙).

The treatment of hypophysectomized animals receiving DCA with STH or with STH and thyroxin did not result in a percentage of lesions comparable to that seen in the intact DCA-treated rats.

Histologically, the kidney, heart, and mesenteric vessels were studied. We will not give a detailed report here of the lesions seen, as they have already been described by several authors.[10-12]

In the *kidney* of DCA-treated intact rats, numerous lesions were seen, of which several examples are given in Figs. 3, 4, 7, 8, 9, and 10. In hypophysectomized animals similarly treated, these lesions could not be found. In slides stained with the PAS technique, it could be seen that hypophysectomized DCA-treated animals showed in the kidney some irregularity of the glomerular basement membranes, but the tubules and arteries, except for a slight dilatation of the former, appeared normal. The disappearance of the renal lesions after hypophysectomy is rapid (Figs. 5 and 6 illustrate this point). Treatment with STH and thyroxin did not modify the picture. In some of the glomeruli of LAP + ACTH-treated animals, lesions could be seen; the general evaluation nevertheless was not very different from that of the hypophysectomized animals.

In the *heart,* the main changes after hypophysectomy were foci of lesions which were less numerous and contained less hyaline material and more cells than controls similarly treated with DCA. However, the effect is not as dramatic as in the kidney (Figs. 11 through 14).

In the *vessels,* lesions of periarteritis nodosa were seen. In addition to the lesions already described, three DCA-treated intact animals of the whole series demonstrated one lesion not previously described—the presence of aneurysm formation in mesenteric vessels (Fig. 15). Aneurysm formation is a common feature in human cases of periarteritis nodosa. We have seen aneurysms for the first time in animals receiving DCA for several months, and this prompted us to re-examine our material. In this series, as well as in others in which DCA has been given for periods of time close to 1 month, aneurysms are not infrequently found when searched for.

Hypophysectomy has the same effect on periarteritis nodosa as on myocarditis in that it diminishes the frequency of the lesions and they have less hyaline material. Nevertheless, as illustrated in Fig. 16, the disappearance of the hyaline material after hypophysectomy is not as quick in extrarenal vascular lesions as in the kidney.

DISCUSSION

It has been shown that hypophysectomy inhibits the development of DCA hypertension.[4] The hypophysis is necessary for the maintenance of the so-called metacorticoid hypertension.[5] Considering the evidence of the present experiments, the presence of the hypophysis would be necessary for the maintenance of DCA hypertension even when the treatment with DCA continues after hypophysectomy.

In renal hypertension, Page and Sweet[13] and Braun-Menendez and coworkers[14] have shown that hypophysectomy lowers the blood pressure, but further tightening of the clamps on the renal artery or administration of

thyroid[13] or ACTH[15] will restore the blood pressure to prehypophysectomy levels. Furthermore, if the constriction is performed weeks after hypophysectomy, then a rise in blood pressure occurs.[13, 16, 17]

The hypertension induced by aortic constriction is inhibited by hypophysectomy.[18] When an aortic constriction is performed in hypophysectomized animals receiving LAP or STH, the blood pressure rises to the level of the nonhypophysectomized controls. A treatment with ACTH does not produce this effect.[19]

In each of these three kinds of experimental hypertension (DCA, renal, and aortic constriction) the hypophysis appears to be differently implicated. Both induction and maintenance of DCA hypertension are dependent on the presence of the hypophysis. The production of renal hypertension is independent of the hypophysis, and its maintenance is in some cases related to adrenocortical function. One can speculate that the discrepancy in this effect on the maintenance is related to some change in the pathogenesis of the renal hypertension with time. The hypertension induced by aortic constriction would be dependent on the presence of STH and more or less independent of adrenocortical function.[20] It seems safe to speculate that the pathogenesis of the three types of hypertension discussed is different. Furthermore many points are still obscure in the pathogenesis of DCA hypertension. In one experiment, we have been able to obtain hypertension with DCA in hypophysectomized rats,[6] but it was not possible to repeat this result. Since DCA hypertension is accompanied by lesions in heart, kidney, and vessels, it is interesting to note that in the sole experiment where hypertension was obtained in hypophysectomized animals, no lesions could be found. It should be said also that in hypophysectomized animals receiving DCA, treatment with STH, LAP, or thyroxin does not induce hypertension or pathologic lesions, except in the case of the doubtful results with LAP and ACTH in the present series. More work is evidently necessary before arriving at definite conclusions.

It is interesting to note the favorable influence of hypophysectomy on the clinical state, hypertension, and pathologic syndrome induced by DCA. It should be recalled that in our experiments, the difference between the renal lesions seen on Day 0 and the day of autopsy was only quantitative. Furthermore, it is the opinion of Rosenberg and associates,[21] with which we agree, that if it is true that the effect of DCA as regards pathologic lesions is different from one experiment to the other, nevertheless the results are quite uniform in a given experiment. Taking this into consideration, the uniform difference in the degree of lesions between intact and hypophysectomized animals treated with DCA is very significant. We see a direct correlation between hypophysectomy and the disappearance of hyaline material and lesions from the kidney. As soon as 12 days after extirpation of the hypophysis, the aspect of the kidney is surprisingly normal. It would take a more detailed histologic study than the present to ascertain the fate of the renal lesions after hypophysectomy. As for the cardiac lesions and vascular lesions outside the kidney, the favorable influence could be explained on the ground that hypophysectomy arrests the appearance of new lesions.

The present experiments prove that established lesions of experimental hypertension can be favorably influenced by hypophysectomy.

SUMMARY AND CONCLUSIONS

Rats unilaterally nephrectomized and drinking 1 per cent NaCl were rendered hypertensive by the administration of DCA. Under these conditions and in spite of the continuation of the treatment with DCA, extirpation of the hypophysis induced: (1) a fall in blood pressure to near normal values; (2) a reduction of renal, cardiac, and vascular lesions. The possible interpretation of these results is discussed.

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THE LYMPHOCYTE RESPONSE TO VARIOUS STRESSORS

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IT HAS been established recently that appreciable numbers of lymphocytes from stressed, adrenalectomized mice present the morphologic alterations of increased cytoplasmic-nuclear ratio, poorly basophilic cytoplasm, and cellular distortion. These cells have been termed stress lymphocytes and appear in increased numbers as a result of a nonadrenocortical mediated response to stress.[1, 2] Lymphocytes subjected to an excess of adrenocortical hormones undergo cytoplasmic budding, pycnosis, and karyorrhexis.[3] It has been postulated that two opposing factors regulate the number and morphologic appearance of circulating lymphocytes. Adrenocortical secretions tend to decrease numbers of normal lymphocytes, whereas nonadrenocortically mediated factors (as yet unidentified) tend to increase numbers of stress lymphocytes. Therefore, a numerical and morphologic evaluation of circulating lymphocytes may quantitatively reflect the balance between adrenocortically mediated and nonadrenocortically mediated effects of stress stimuli.

The quantitative and qualitative effects of irradiation, bacterial pyrogens, and histamine on the lymphocyte response of intact and adrenalectomized animals were studied.

MATERIALS AND METHODS

One hundred male CBA strain mice, 12 to 16 weeks of age, were used. Adrenalectomy was performed under ether anesthesia by the usual dorsal approach 24 hours prior to stress.

Tail blood samples for total leukocyte and differential counts were taken prior to each experiment and at various time intervals after the particular stressor was administered. Blood films were stained with May-Grünwald Giemsa.

A constant DC 250 kv. machine with a 0.5 mm. copper and 1 mm. aluminum filter was used for animals given whole-body irradiation. The bacterial pyrogen (Piromen PV Baxter) and histamine were diluted with 0.85 per cent pyrogen-free saline and administered on the basis of dose per 20 grams body weight.

The following experimental procedures were performed: (1) 10 adrenalectomized mice were given 0.25 ml. pyrogen-free saline intravenously and blood samples were taken at 0, 3, and 6 hours, and 1, 3, and 8 days; this group served as a control for the treated, adrenalectomized groups; (2) 10 intact mice and 10 adrenalectomized mice were given 50 r. whole-body irradiation per mouse and blood counts performed at ½, 1, 2, and 4 hours, and 6, 13, and 21 days; (3) four groups of 10 adrenalectomized mice each were given bacterial pyrogen in doses of 6.25 γ, 5.0 γ, 4.25 γ, and 2.5 γ, respectively. Blood counts on each group were performed at 0, 3, and 6 hours, 1, 2, 5, 8, and 11 days after the single intravenous injection; (4) two groups of 10 adrenalectomized mice were given histamine (250 and 500 mg. per kilogram, respectively) and blood counts were performed 0, 3, and 6 hours and 1, 3 and 8 days after intravenous injection.

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RESULTS

Fig. 1 demonstrates the lymphocyte response of adrenalectomized mice following a single injection of 0.25 c.c. pyrogen-free saline. (In this and successive graphs the bars represent the total number of lymphocytes, the hatched portions the number of stress lymphocytes, and the clear portions the number

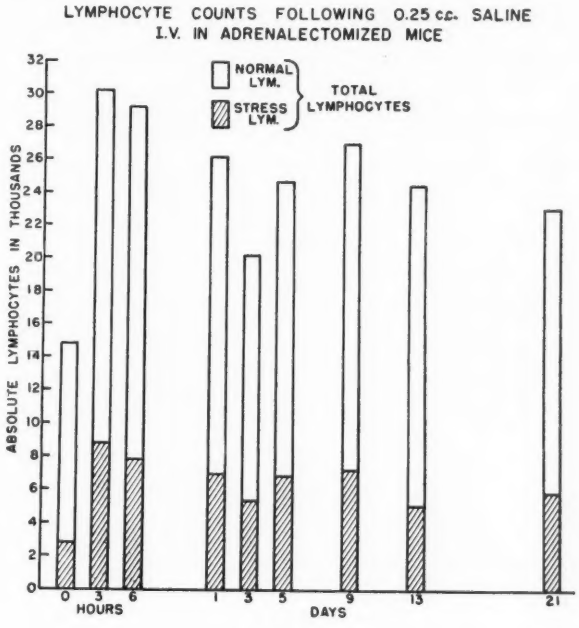
LYMPHOCYTE COUNTS FOLLOWING 0.25 c.c. SALINE
I.V. IN ADRENALECTOMIZED MICE
NORMAL LYM.
STRESS LYM.
TOTAL LYMPHOCYTES
ABSOLUTE LYMPHOCYTES IN THOUSANDS
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
0 3 6
HOURS
1 3 5 9 13 21
DAYS

Fig. 1.

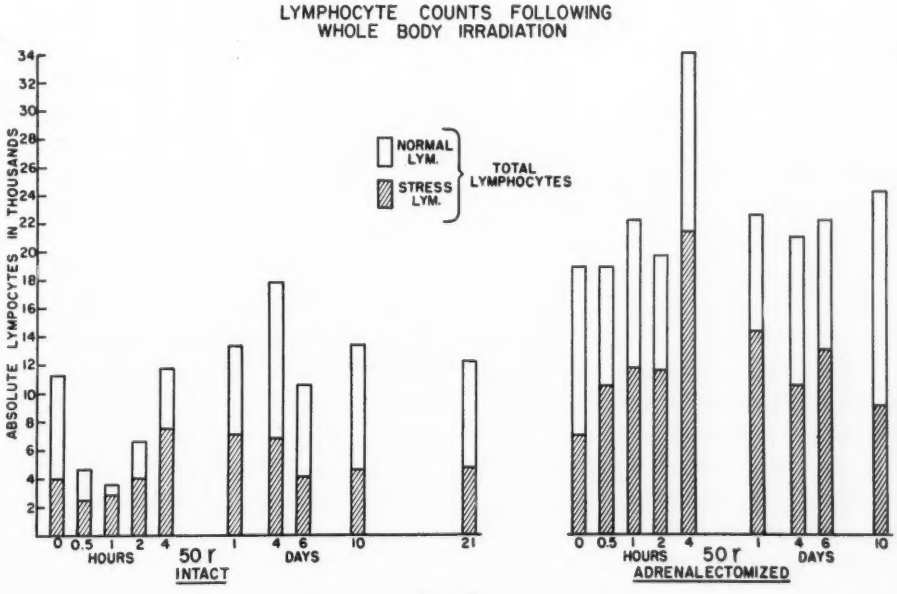

Fig. 2.

of normal lymphocytes.) Three and six hours following the injection of saline the number of both normal and stress lymphocytes increased significantly. In the ensuing period, the levels of both normal and stress lymphocytes decreased slightly and thereafter remained relatively stable.

The lymphocyte responses of intact and adrenalectomized mice following 50 r. whole body irradiation are presented in Fig. 2. The number of normal lymphocytes in intact animals decreased by one-half hour and remained at low values for four hours, whereas stress lymphocytes were increased at four hours.

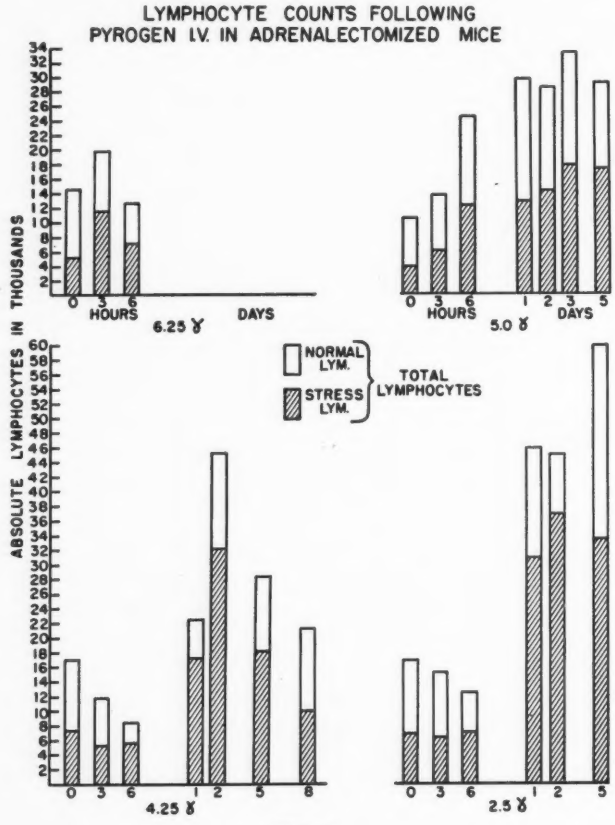

Fig. 3.

Control values were regained on the fourth day. In the adrenalectomized animals the number of stress lymphocytes increased steadily to 22,000 per cubic millimeter by four hours and thereafter remained at significantly high levels as compared with the adrenalectomized, saline-treated controls (Fig. 1). No decrease in the number of normal lymphocytes was found.

All doses of bacterial pyrogens employed (Fig. 3) abolished the initial 3- and 6-hour lymphocytosis found in the saline controls (Fig. 1). However, a lymphocytotic response, primarily due to an increase in numbers of stress lymphocytes, was found at one day in the 5.0 γ, 4.25 γ, and 2.5 γ dose groups. As the dose was decreased, the number of stress lymphocytes increased so that

an inverse dose-response relationhip was found within the dose range employed. Mice which received 6.25 γ did not survive beyond six hours.

Lymphocyte responses following intravenous administration of 250 mg. per kilogram of histamine and 500 mg. per kilogram of histamine to adrenalectomized mice are compared in Fig. 4. An increase in the number of stress lymphocytes (which was significantly greater than that found in the saline controls) occurred during the first six hours in the group which received 250 mg. per kilogram. Control values were attained by the first day. The number of stress lymphocytes of the groups which received 50 mg. per kilogram was not found to be increased significantly until the first and third days.

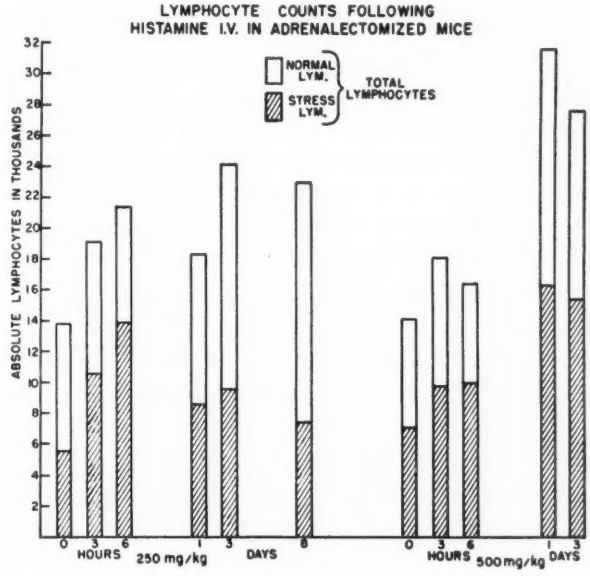

Fig. 4.

DISCUSSION

It is apparent from data presented here that normal and stress lymphocytes respond differently depending on the type and concentration of the stressor employed and whether the adrenals are present. The usual nonspecific response of blood lymphocytes to stress stimuli as determined on adrenalectomized animals is the production of a lymphocytosis greater than that characteristic for nonstressed adrenalectomized mice. The mild stress involved in the administration of pyrogen-free isotonic saline resulted in a slightly greater lymphocytosis (both normal and stress lymphocytes) than that following adrenalectomy alone. However, no progressive increase was found in the ensuing period.

The lymphocyte response following irradiation is obviously altered by the well-known sensitivity of lymphatic tissue to direct destruction by irradiation. From data presented here it is apparent that in intact animals the normal lymphocytes are more susceptible to irradiation and ensuing adrenocortical mediation than are stress lymphocytes. Normal lymphocytes were not decreased

in number following whole-body irradiation of adrenalectomized mice, and the number of stress lymphocytes was increased at the time intervals studied.

A specific response to pyrogen is that of a markedly increased number of stress lymphocytes with the lower doses. Higher doses of pyrogen did not produce increased numbers of stress lymphocytes. A similar observation has been noted following the administration of graded amounts of epinephrine with the result that the period necessary for lymphocytosis to appear following higher doses was prolonged compared with lower doses.[4] Histamine administration resulted in lymphocytosis; the higher dose induced a greater, although delayed, increase in stress lymphocytes.

SUMMARY

The quantitative and qualitative effects of irradiation, bacterial pyrogens, and histamine on the lymphocyte responses of intact and adrenalectomized mice were studied.

In irradiated intact mice normal lymphocytes rapidly decreased in number, whereas stress lymphocytes increased by four hours. Stress lymphocytes in irradiated adrenalectomized mice increased remarkably. Normal lymphocytes are more susceptible to direct destruction by x-ray in combination with x-ray induced increased adrenocortical secretion than are stress lymphocytes.

Within the dose range of bacterial pyrogens employed, the number of stress lymphocytes increased as the dose was decreased. The overwhelming toxicity of pyrogen in higher doses probably prevented the lymphocytotic response.

Histamine administration resulted in a dose-response increase of stress lymphocytes.

The lymphocytosis of stress lymphocytes which occurs in stressed adrenalectomized mice reaffirms the presence of an as yet unknown lymphocytopoietic promoting mechanism.

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STUDIES OF LEUKOPOIESIS

The Technique of Leukopheresis and the Response of Myeloid Tissue in Normal and Irradiated Dogs

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THE rate of growth of myeloid tissue is a subject which has eluded definition in the same precise terms available for erythroid tissue. Such fundamental data as the survival time of the myeloid leukocytes, the rate of production of these cells, and the duration of their stay in the marrow, the peripheral blood, and the tissues have been gained slowly and are still controversial.

Much of the difficulty in delineating the physiology of leukopoiesis and myeloid leukocytes stems from the multiplicity of factors affecting the level of these cells in the peripheral blood. Acute and marked fluctuations in leukocyte level occur on the basis of sequestration in or release from various segments of the vascular system. The spleen[1, 2] and lung[3, 4] appear to play a particularly prominent role in such acute changes, probably because of the peculiar architecture of the vascular bed of the former and the tremendous surface area of the latter. The influence of various substances such as adrenalin and histamine on the blood leukocyte level indicates the relationship of the physiologic state of the vascular tree to the blood leukocyte count. Leukopenia also occurs as a result of "sludging" or sticking of leukocytes to endothelial surfaces following such phenomena as adminstration of typhoid vaccine or various macromolecular substances.[5, 6]

Since the leukocyte possesses its own motility and can move freely outside of the vascular system, the number of these cells within the body at a given moment cannot be determined. The continual migration of leukocytes into various body lumina is well known.[7] Osgood[8] has estimated the number in the tissues to be some 40 times that within the vascular compartment. Furthermore, the huge number of leukocytes rapidly accumulating at the site of an infection and the continuous supply of these cells to such an area attest the ability of the myelopoietic system to furnish leukocytes at a greatly accelerated rate. The "turnover" of leukocytes in the peripheral blood under such circumstances must be much greater than under more basal conditions.

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From such observations it is clear that one cannot look upon sudden changes in peripheral blood leukocytes as indicative of changes in rate of growth unless the other factors affecting the leukocyte level can be quantitated or controlled. Persistent or prolonged changes in blood leukocyte level, when correlated with morphologic changes in the cells of the blood and marrow, usually indicate whether leukocyte production is increased or decreased in relation to normal. However, no finer degree of quantitation is possible unless special techniques are used. Furthermore, the diffuseness and variability in cellularity of the marrow organ, which render estimates of its size difficult, further complicate the study of leukopoiesis. The marrow contains a mass of mature myeloid leukocytes, which is considerable and may be much larger than the mass of leukocytes normally present in the peripheral blood. Therefore, one must consider leukocyte production in terms of release of cells from the marrow organ and actual replacement of such cells by growth processes.

It is well known that the capacity of the marrow for red blood cell production can increase 6 to 8 times upon demand.[9] The rapid alterations in the marrow in response to certain infections indicate the rapidity with which leukopoiesis can accelerate. The size of the tissue responsible for leukopoiesis and the variability of its productive capacity must be borne in mind in attempting to assess turnover rate, survival time, and alterations in peripheral blood white cell level. It is possible that the lifespan of leukocytes may vary widely under different conditions, just as the rate of production of these cells varies.

Most of the available data on leukocyte survival time are derived from methods of cross circulation or transfusion or isotopic labeling. The animal and human experiments involving cross transfusion of normal or leukemic cells into normal or irradiated recipients[10-12] are subject to several criticisms. The most cogent of these are the possibility of immunologic and genetic incompatibility between the donor cells and the recipient, and other subtle factors which might yield erroneous results due to nonspecific removal of cells from the circulation by clumping, "sludging," or sequestration. The presence of disease or tissue damage in the recipient, as was the case in some studies, further obscures the results. Furthermore, as Ottesen[13] suggested, the time spent within the circulation may represent only a fraction of the total lifespan of leukocytes.

Methods of estimating white cell survival and turnover based on the rate of desoxyribose nucleic acid[13-17] synthesis are appealing since the criticisms relative to cross-transfusion methods are largely obviated. However, all aspects of this approach are not established beyond question. Most workers have employed radioactive phosphorus, partitioning the nucleic acid phosphorus by modifications of the Schmidt-Thannhauser procedure. This method has been criticized as to the specificity of the results.[18] Furthermore, the administration of high doses of P^{32} as a means of giving the tag, as employed by some,[14, 15] might obscure the data on nucleic acid synthesis since ionizing radiation markedly depresses this process. The results of Kline and Cliffton[15] indicated an average lifespan of some 13 days for leukocytes in the normal human being. (Lymphocytes and myeloid forms were not differentiated in this study.) It was concluded

that the leukocytes remained in the marrow for 4 days of this 13-day period. Ottesen[13] calculated the lifespan of normal human myeloid leukocytes to be about 20 days, with an intramedullary phase of 5 to 6 days.

The present experiments were designed to study the capacity of the marrow to provide myeloid leukocytes to the peripheral blood in response to the rapid removal of these elements. Large numbers of white cells were cleared from the peripheral blood without the introduction of factors which would lead to marrow damage or sequestration of cells within various vascular compartments, since only the animal's own blood was used in most instances. By quantitating the numbers of cells removed and the rate of replenishment of leukocytes into the peripheral blood of normal and irradiated dogs, data in regard to the functional capacity of the marrow for leukopoiesis should be obtainable. This approach was patterned after the classical work of Robscheit-Robbins and Whipple,[19, 20] in which erythropoiesis was studied by observing the response of the marrow to quantitative removal of red cells.

Platelets were removed with the leukocytes, and varying degrees of thrombocytopenia were induced. The data in regard to platelets are the subject of a separate report.

Lymphocytes, monocytes, and other types of white cells were, of course, removed during the procedure. The vast majority of cells removed, however, were myeloid. Lymphocytes disappeared rapidly in most instances and were relatively depressed during the leukocytosis subsequent to leukopheresis. No attempt has been made to quantitate the response of lymphoid tissue to the procedure, the data applying to myeloid leukocytes only.

EXPERIMENTAL METHODS

Mongrel dogs varying in weight from 20 to 50 pounds were used. These animals were in good health and nutritional status. All were observed for a period of two weeks or more before being used experimentally. Those dogs subjected to preliminary venesection were given supplementary iron in their diets.

The animals were anesthetized with pentobarbital intravenously, and the femoral artery and vein on one side exposed. The artery was cannulated with a silicone-coated glass cannula or polyethylene tubing with a side-arm attachment for pressure recording. The vein was similarly cannulated. The artery and vein were connected to syringe systems which permitted removal and injection of measured volumes of blood. Serial base-line cell counts were made from arterial blood for at least one hour. Blood volume was determined by the Evans blue dye method. Each animal received 600,000 units of procaine penicillin and 250 mg. streptomycin intramuscularly at the beginning of the experiment only. Blood was removed in 40 to 50 ml. aliquots and placed in plastic centrifuge tubes containing 10 per cent ethyelene diamine tetra acetate disodium* in 0.3 ml. amounts. In those experiments where dextran† was employed, 45 ml. of blood was mixed with 5 ml. of 6 per cent dextran and allowed to sediment for 15 minutes. The plasma containing white cells and platelets was then centrifuged at 3,000 rpm for 15 minutes, and the cell-free plasma removed and reconstituted with the red cells. In this way almost quantitative removal of white cells and platelets was achieved.

In those experiments where no dextran was employed, plasma was aspirated from above the buffy coat after centrifuging at 2,800 rpm for 15 minutes in a standard centrifuge. The buffy coat was removed en masse along with the upper layer of red cells by rapid

*Sequestrene—Alrose Chemical Company.

†Pharmacia Laboratories Dextran, av. molecular wt. 120,000 and intrinsic viscosity 0.40.

aspiration using a suction head which closely approximated the diameter of the tube, so that the entire layer was removed at the same time. The red cells thus removed with the white cells and platelets could be separated at a later time and readministered. The cell-free plasma and the red cells were reconstituted and readministered to the animal. Counts of white cells and platelets on blood so handled indicate removal of 85 to 90 per cent of the white cells and platelets. The cells collected were kept in siliconed containers in ice until used. Total counts on the cell collections were made in quadruplicate after thorough mixing.

Although separation of the white cells and platelets achieved by dextran was very efficient, the plasma containing dextran, when readministered to the dogs, caused blood volume alterations and in some instances anaphylactic-type symptoms with profound leukopenia. Blood dextran levels were followed and in no instance did the concentration exceed that attained in the clinical use of the material.[21] Not all animals were adversely affected, but because of the unpredictable effects no dextran was employed in the majority of the experiments.

Sequestrene in large enough dosage to cause profound hypocalcemia (4 to 5 mg. per cent serum calcium) induced no significant change in the white cell level in two control experiments. However, several animals were lost as a result of electrolyte imbalance brought about by Sequestrene. In later experiments this was avoided by more precise replacement therapy with calcium and magnesium. The amount of Sequestrene employed did not much exceed the theoretical minimum required to decalcify the blood in the collection tubes. However, because of the delayed renal excretion of Sequestrene, a gradual cumulative effect occurred. This was counteracted in all the later experiments by slow intravenous administration of calcium gluconate and magnesium sulfate at a rate roughly equal to the rate Sequestrene was used. Only one animal was lost on this basis after this routine was initiated.

The electrocardiographic changes associated with leukopheresis were an essential guide for prevention of cardiovascular collapse. These changes are of particular interest in regard to electrolyte imbalance. In most animals rendered hypocalcemic, characteristic[22] prolongation of the Q-T interval, depression of T wave (or inversion), and depression of P-wave voltage were observed. These findings are similar to those described by Nakasone and his associates[23] resulting from rapid transfusion of citrated blood into dogs. The changes were preventable and/or reversible with calcium gluconate and magnesium sulfate intravenously. (The combination was more effective than calcium alone, as would be expected since Sequestrene chelates all divalent cations to varying degrees.)

In this manner the animal's blood volume was removed, depleted of white cells and platelets, and returned to the animal as a continuous operation.* The initial deficit of blood outside of the animal was made up in some instances by sterile blood collected from the same animal during the previous two-week period and stored in ACD at refrigerator temperatures. In other animals, the initial deficit incurred during the exchange before the removed blood had been processed for return was partially compensated by Ringer's solution. It was found that a considerable volume deficit (250 to 500 ml. of whole blood) was well tolerated.

Sterile instruments, syringes, plastic centrifuge tubes, and equipment were used throughout, although no attempt was made to handle the blood in an aseptic manner. Antibiotics were administered as described. Blood cultures were taken from 5 dogs during and immediately after the procedure and none showed bacterial growth. Anerobic cultures were not made. None of the animals reported herein developed wound infection. Although the possibility of bacterial contamination cannot be entirely excluded, it is not likely that this was a factor in the hemopoietic changes observed.

Base-line blood volume determination was by the Evans blue dye method, employing a Coleman Jr. spectrophotometer. White cell and red cell counts and hematocrit determinations were made from freely flowing arterial blood drawn into the appropriate amount of balanced oxalate and were performed immediately upon sampling. Bureau of Standards certified pipettes were used for counts and Wintrobe tubes for hematocrit determination. Duplicate white counts were not made unless an apparent discrepancy occurred. Repeated serial counts at frequent intervals reduced to a minimum the possibility of sampling or

*The procedure is denoted "leukopheresis" subsequently in this paper.

counting error. White cell counts at leukopenic levels were obtained from arterial, venous, and capillary blood and were performed under high-power magnification to reduce the possibility of encountering artifacts. Blood smears for differential counts were made from fresh blood at each time of sampling. Bone marrow differential counts were based on 500 nucleated cells. Imprint preparations from punch biopsies were made in some animals in an attempt to estimate the degree of cellularity of the marrow.

The dogs exposed to x-ray were given 250 r whole body irradiation* as follows: each animal was exposed to 250 r delivered at 250 kv. and 15 Ma. Filters used were as follows: 0.21 copper inherent, 0.5 copper parabolic, and 1.0 aluminum. The 250 r dose required approximately 12 to 15 minutes to deliver at a distance of 100 cm. from each of two x-ray heads above and below the center of the animal, which was in a prone position. All calibrations were corrected for temperature and atmospheric pressure. These animals appeared to be in good health at the time of the exchange procedure. All animals continued to eat well and exhibited no diarrhea or weight loss during the first three postirradiation days.

EXPERIMENTAL RESULTS

I. *Hematologic Changes in Normal Dogs Subjected to One Leukopheresis.*—

Production of leukopenia: Seventeen dogs were subjected to leukopheresis over the period of a single day. Fifteen of these dogs became leukopenic within a few hours. Typical experiments are shown in Figs. 1 to 3. Of the other 2, leukopenia was not induced in 1 and only after prolonged exchange in the other; these results will be discussed subsequently. Although the amount of blood cleared of leukocytes per hour varied widely in earlier experiments, it became clear that leukopenia (amounting to 2,000 cells per cubic millimeter or less) occurred in all animals in which leukopheresis was performed at a rate of 1.5 blood volumes or more per hour for 3 hours. Slower rates of clearance (e.g., 1 blood volume per hour) yielded variable results and would not always produce marked leukopenia even though continued long enough to remove many more cells than those originally circulating.

Estimates of the number of white cells removed were made by carrying out multiple counts of the cell collections. Such estimates are undoubtedly low because of unavoidable sources of loss incident to collection. However, it will be noted from Table I that in most instances the number of cells removed does not differ widely from the number originally circulating. In Dogs 1 and 10 about twice the number originally circulating were removed. In the case of Dogs 5b and 7, however, many more cells were removed.

The results of Dogs 5b and 7 are depicted in Figs. 4 and 5. Both of these animals had large blood volumes and body mass. Both were leukopheresed at a relatively slow rate in terms of the number of blood volumes cleared of leukocytes per hour. In Dog 5b an estimated 6.1×10^{10} white cells were removed over a ten-hour period. Since the peripheral blood leukocyte level remained fairly stable during this time, this would mean the cells were entering and being removed from the circulation at a rate of about 0.6×10^{10} cells per hour. This number would amount to two-thirds of those cells originally circulating.

In Dog 7 (see Fig. 5) the peripheral blood count was lowered from 11,000 per cubic millimeter to 4,100 per cubic millimeter after 5.5 hours' depletion at a

*The irradiation was carried out in the Radiation Laboratory, Atomic Energy Commission, U. C. L. A. We are indebted to Dr. L. R. Bennett and Mr. Raymond B. Meek for this portion of the work. This dose of 250 r as delivered from two heads has been found to be approximately an $L.D._{50}$ for dogs, and compares with 350 to 400 r delivered from a single head.

rate of 1.0 blood volumes per hour. The level remained between 4,000 and 7,300 per cubic millimeter for 3 more hours during leukopheresis at the same rate. When the rate of clearance was increased to 1.52 blood volumes per hour, the white cell count fell to 1,800 per cubic millimeter in about three and one-half hours. These two animals represent instances of slow leukopheresis without the

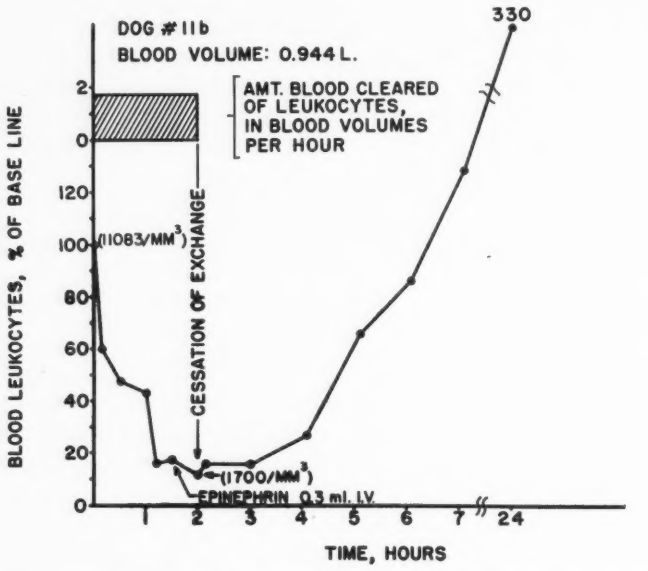

Fig. 1.—A typical experiment in a normal dog showing the alterations in leukocyte count incident to a single sustained leukopheresis. Note the period of sustained leukopenia following cessation of leukopheresis.

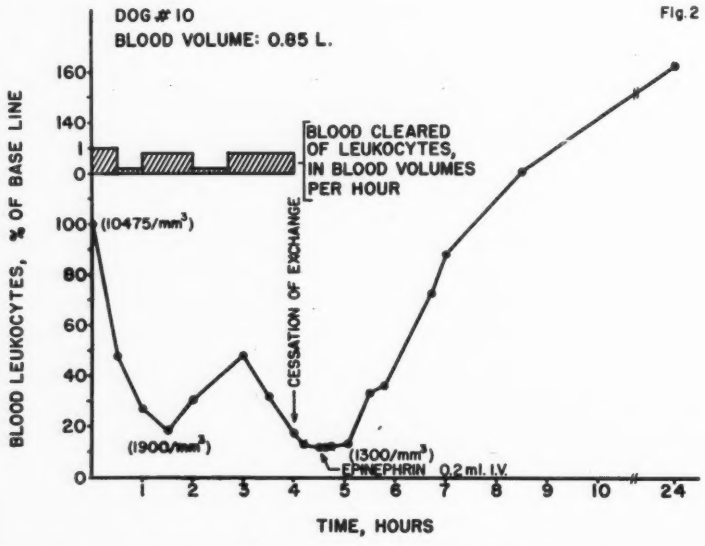

Fig. 2.—A normal dog leukopheresed at a variable rate. Note the failure of epinephrine to affect the leukopenia after cessation of leukopheresis.

induction of marked leukopenia. They emphasize the necessity of rapid, massive removal of white cells for the production of leukopenia by this means.

It is apparent that the procedure of leukopheresis brings about a change in the rate of entry of myeloid cells into the circulating blood. This is illustrated by the results in Fig. 3, where, after recovery to nearly 100 per cent following leukopheresis, removal of cells was reinstituted. The slope of the recovery curve after this second leukopheresis indicates a rise in circulating white cells at a rate of 0.69×10^{10} per hour, as compared to 0.25×10^{10} per hour during the first recovery period.

In several instances intravenous epinephrine was given during the period of leukopenia which followed leukopheresis. Sufficient amounts (0.1 to 0.5 ml.

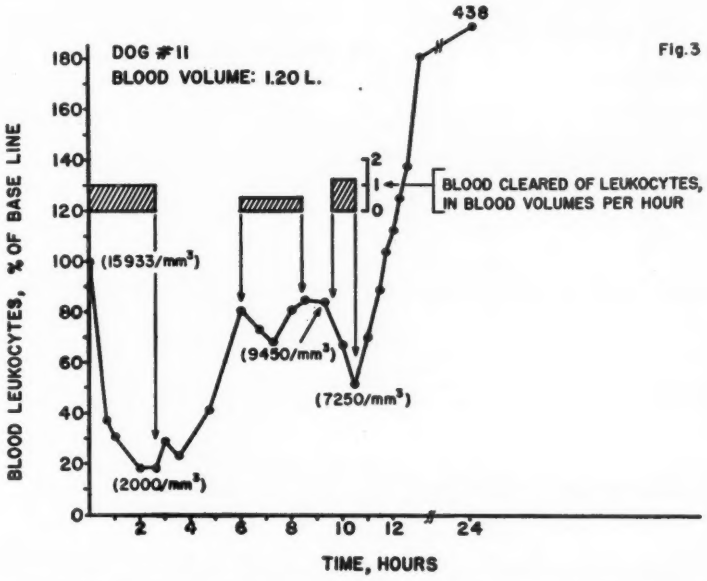

Fig. 3.—The effect of discontinuous leukopheresis on the rate of return of leukocytes into the peripheral blood. Note the acceleration of rise in blood leukocytes after the last leukopheresis as compared to the initial response.

TABLE I. ESTIMATE OF TOTAL CIRCULATING WHITE CELLS PRIOR TO EXCHANGE, REMAINING AT END OF EXCHANGE, AND TOTAL NUMBER REMOVED BY LEUKOPHERESIS

DOG NUMBER	CIRCULATING WHITE CELLS PRIOR TO EXCHANGE	CELLS REMOVED BY EXCHANGE	CELLS REMAINING
1	1.2×10^{10}	2.6×10^{10}	0.4×10^{10}
5	0.54×10^{10}	0.48×10^{10}	0.2×10^{10}
8	1.5×10^{10}	1.5×10^{10}	0.015×10^{10}
13a	0.95×10^{10}	0.61×10^{10}	0.2×10^{10}
10	1.5×10^{10}	2.4×10^{10}	0.43×10^{10}
14b	1.17×10^{10}	0.9×10^{10}	0.15×10^{10}
8b	0.96×10^{10}	0.96×10^{10}	0.24×10^{10}
13b	1.2×10^{10}	1.23×10^{10}	0.2×10^{10}
14c	1.04×10^{10}	1.0×10^{10}	0.15×10^{10}
11b	1.04×10^{10}	1.0×10^{10}	0.16×10^{10}
5b	0.93×10^{10}	6.1×10^{10}	0.35×10^{10}
7	1.6×10^{10}	5.8×10^{10}	0.56×10^{10}

of 1/1,000 solution) to produce marked tachycardia, arrhythmias, and blood pressure elevation failed to cause any significant change in the white cell level, counts being made at 1-minute intervals for 15 minutes. Once a marked leukopenia had been induced, multiple blood counts were taken from different sites

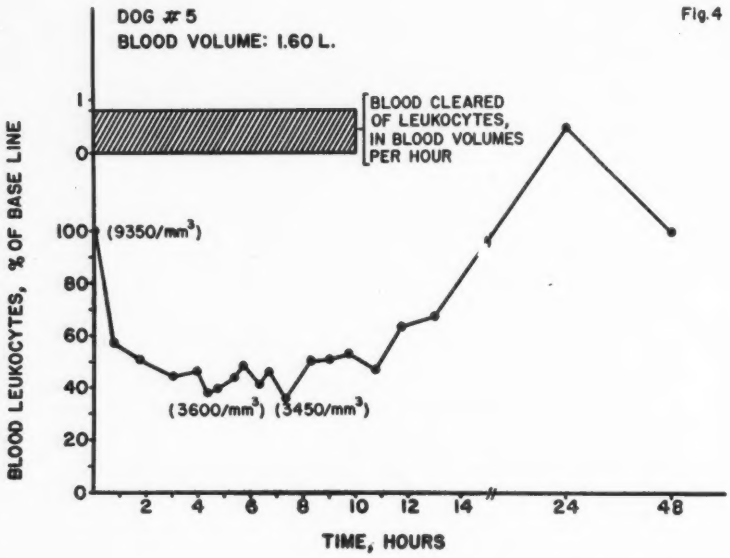

Fig. 4.—The failure of sustained, continuous leukopheresis at a relatively slow rate to induce leukopenia; 6.1×10^{10} white cells were removed during the procedure.

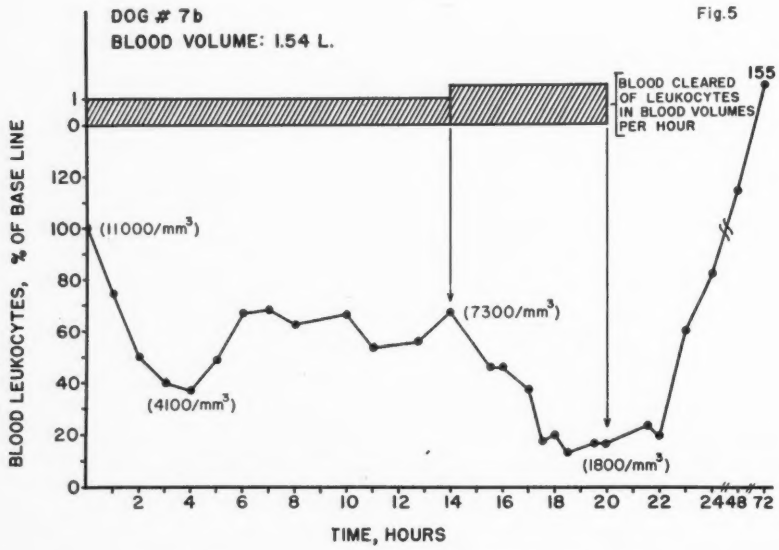

Fig. 5.—Failure of leukopheresis to induce leukopenia until the rate of clearance was accelerated. Note the delay in recovery of blood leukocytes after this prolonged but continuous leukopheresis.

such as the femoral vein, vena cava, abdominal aorta, brachial vein, and skin of the ear. These were compared with the values obtained from the femoral artery and in all instances agreed closely.

On the basis of these observations, it is felt that a distribution or sequestration type of leukopenia was unlikely. The possibility of "sludging" and clumping of the leukocytes in the capillary bed seems improbable in view of the similar results with arterial, venous, and capillary blood. The omental circulation of one dog was observed microscopically. During the period of leukopenia no evidence of white cells adhering to the capillary wall was noted. Indeed, very few leukocytes could be seen.

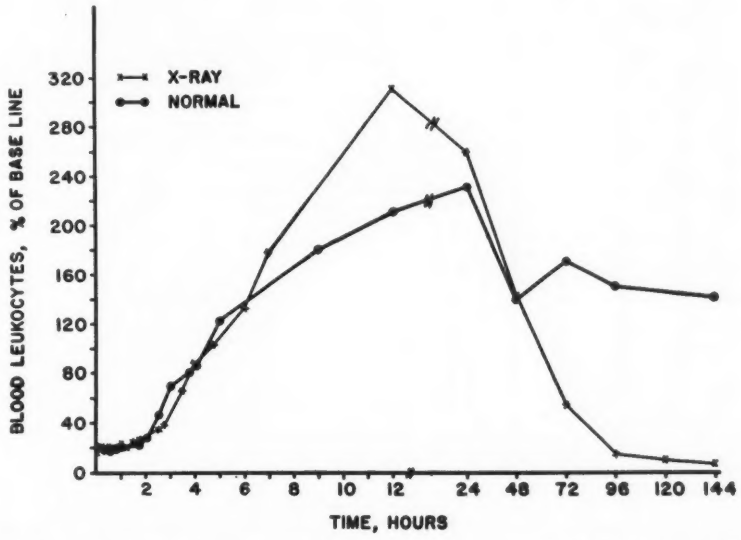

Fig. 6.—Average values in 9 normal and 3 irradiated dogs ($L.D._{50}$ whole body) after a single leukopheresis. The zero point is the time of cessation of leukopheresis for both groups.

Rate of return of leukocytes in the peripheral blood of normal animals after production of leukopenia: Adequate data are available in 15 normal dogs in regard to the recovery phase. Fig. 6 shows the average values in 9 of these animals expressed in per cent of the initial level before leukopheresis. The results in the other animals were similar, except for the 2 previously discussed, which were excluded from the average group. It can be seen that the animals were back to the 100 per cent value in approximately four and one-half hours. The actual range of values at this time varied from 90 to 132 per cent. The lag phase of some 30 to 90 minutes before the rapid rise in leukocyte level began is noteworthy and will be commented upon later.

Fig. 7 shows the same data expressed in numbers of cells per cubic millimeter. The slope of the curve is much steeper and approximates the reciprocal of the slope of the downward curve for the production of the leukopenia. The abruptness of the rise is quite striking and was similar in every animal. Every animal

had reached a level of 5,000 white cells per cubic millimeter in 2 to 3 hours, the majority within 2 hours. The curve after the initial lag period before the level began to rise is essentially a straight line for 6 to 7 hours after the end of the first postexchange hour. Between postexchange hours 2 and 3, there is an increment in the white count from 5,200 to 9,000, or 3,800 cells per cubic millimeter.

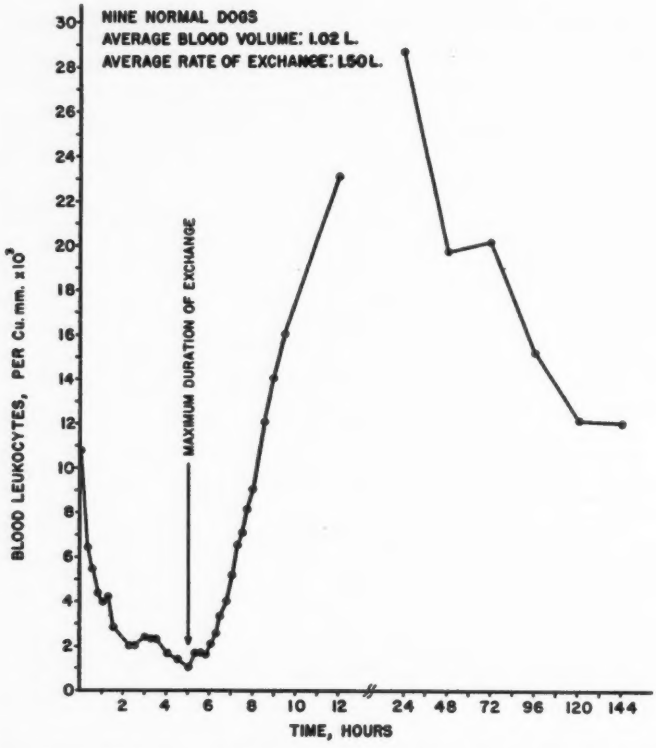

Fig. 7.—Average values in 9 normal animals subjected to a single leukopheresis. Leukocytes expressed as numbers per cubic millimeter.

On the basis of the average blood volume of 1 L. this represents 0.38×10^{10} cells entering the circulation per hour. The average number of cells present in the circulation initially in this group was 0.8×10^{10}. During the period of recovery, therefore, enough cells entered the circulation in slightly over 2 hours to replace completely those initially present. In some animals the exchange was discontinued immediately upon reaching the maximum leukopenia. In others the exchange was continued for periods varying from 15 minutes to 6 hours. It was noted that regardless of the time that leukopenia was maintained by leukopheresis, a delay was present before the white cell level began to rise.

Differential cell counts were done during the period of rising leukocyte levels. All animals showed a mild shift to the left beginning during the early part of the recovery phase. None showed immaturity of the cell population during the actual exchange period. For the most part the shift at the beginning of recovery involved an increase in stab forms with only a scattering of more

immature forms. However, at 24 hours more metamyelocytic and myelocytic forms were seen. Even at this point, however, the shift to the left was not profound.

In most of the animals the lymphocytes practically disappeared. In 2 there was a relative lymphocytosis during leukopheresis. The lymphocytes usually declined sharply during the period of leukopheresis and remained low throughout the recovery period.

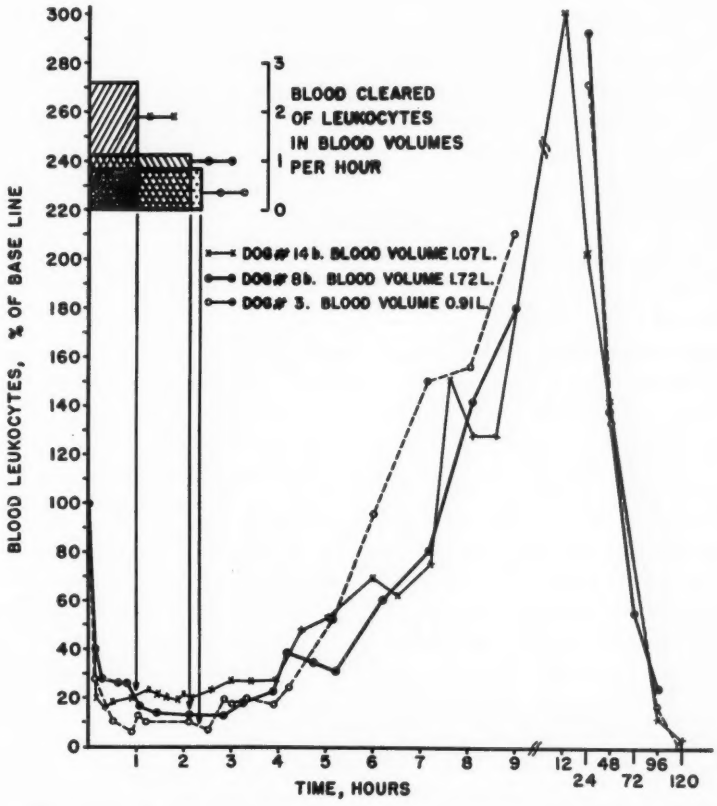

Fig. 8.—Response of blood leukocytes, expressed as per cent change from base line, in 3 irradiated ($L.D._{50}$ whole body) dogs. The animals were exposed to irradiation 24 hours (No. 14b), 36 hours (No. 8b), and 42 hours (No. 3) prior to onset of leukopheresis.

Varying degrees of anemia were produced in most animals incident to unavoidable loss of red cells. However, in 5 animals in which stored red cells were on hand from previous phlebotomies, no anemia was apparent at the end of the acute exchange. (In every case only the animal's own blood was administered so as to avoid any possibility of incompatibility.) The response of the myeloid elements to the procedure was not affected by anemia and bore no relation to the severity thereof.

II. *Hematologic Changes in Irradiated Dogs Subjected to Single-day Leukopheresis.*—The results in 3 irradiated animals subjected to a single white cell

depletion at times varying from 18 to 42 hours after exposure to $L.D._{50}$ whole body x-ray are shown in Fig. 8. The uniform pattern of response is notable.

Production of leukopenia: In each case the initial white cell level was lower than the average for the nonirradiated group. The fall in white cells was much more precipitous than in the control animals. The low point of their leukopenia was reached within one-half hour, despite differences in the rate of leukopheresis (0.8 blood volume per hour in Dog 3, 1.2 blood volumes per hour in Dog 8b, and 2.6 blood volumes per hour in Dog 14b). The fall in white cells began before the circulating blood had been cleared of leukocytes. This is indicative of a slow rate of entry of leukocytes into the circulating blood.

It is noted that leukopheresis was continued in each case beyond the point of maximum leukopenia without further reduction. The extraction ratio of the procedure is not much better than the number of cells present at the lowest point. Consequently, the continued exchange could not have accomplished much since almost as many cells were being returned as removed. This is further substantiated by the fact that each animal began to show return of leukocytes at approximately the same time after the institution of depletion regardless of the duration of the procedure.

Although observations from such a small group of animals lack statistical weight, it is of interest to compare the differences between the two groups. It will be noted (Fig. 9) that the lag phase before the sharp rise in blood leukocytes began is considerably longer in the x-ray group (3 hours) than in the control group (¾ hour). Furthermore, the slope of the upward curve is less than in the normals. From a selected area on the curve after it has straightened out (between postleukopheresis hours 4 and 5), it will be observed that the average increment in white cell level is from 3,300 per cubic millimeter to 5,200 per cubic millimeter (or 1,900 cells per cubic millimeter per hour) in the irradiated group, or the entry of 0.23×10^{10} cells per hour into the peripheral blood. This is in contrast to the entry rate of 0.38×10^{10} cells per hour for the normal group at a similar part of the curve, a difference of at least 0.15×10^{10} cells per hour in the two groups (or 1,500,000,000). This difference would be even more marked if the average blood volumes of the two groups were identical.

Despite the slower rate of return of the leukocyte level in the irradiated group and the markedly longer lag period after cessation of exchange, it is remarkable to what extent these irradiated animals could respond. As is shown in Fig. 6, when the leukocyte levels are expressed in terms of the per cent change from the base-line levels, the irradiated animals recovered at the same rate as the normals. In fact, the early portions of the two curves are identical. After the fifth postexchange hour the irradiated animals exceeded the normal animals in the rate of rise as expressed in per cent. In terms of actual numbers of cells produced, however, Fig. 9 demonstrates the marked superiority of the normal group.

The downward trend in white count in the irradiated dogs is remarkable because of its uniformity, as shown in Fig. 8. Each of these animals received the same dose of x-ray administered in the same way, but the exchange was per-

formed at different times after exposure. Regardless of the time lapse between x-ray and exchange, the development of leukopenia occurred at the same time.

The bone marrow of these dogs was not aplastic when examined prior to the first leukopheresis. Aside from an increased number of degenerated and atypical forms, the only noteworthy change from normal was the relative increase in mature myeloid forms. Bone marrow findings are discussed in detail later in the article.

III. *Hematologic Changes in Dogs Subjected to Leukopheresis on Three Successive Days.*—

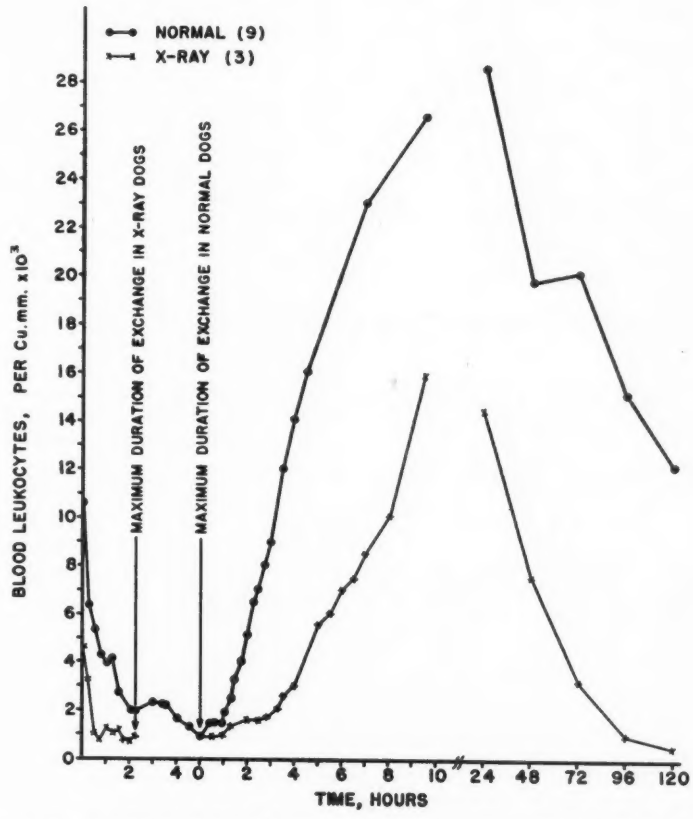

Fig. 9.—Average values of blood leukocytes in 9 normal and 3 irradiated dogs expressed as number per cubic millimeter. The point of cessation of leukopheresis has been approximated for the 2 groups to allow comparison of the recovery phase.

Normals: The pattern of response was determined in animals subjected to repeated leukopheresis in order to increase the demand for leukocytes imposed on the hemopoietic organ. Repeated surgery was obviated by leaving the femoral artery and vein cannulated with flexible polyethylene catheters. Leukopheresis could thus be reinstituted at any time. The normal animals were in good condition at the end of the procedure and subsequently recovered completely. They showed no ill effects from the procedure several months later. Figs. 10 and 11

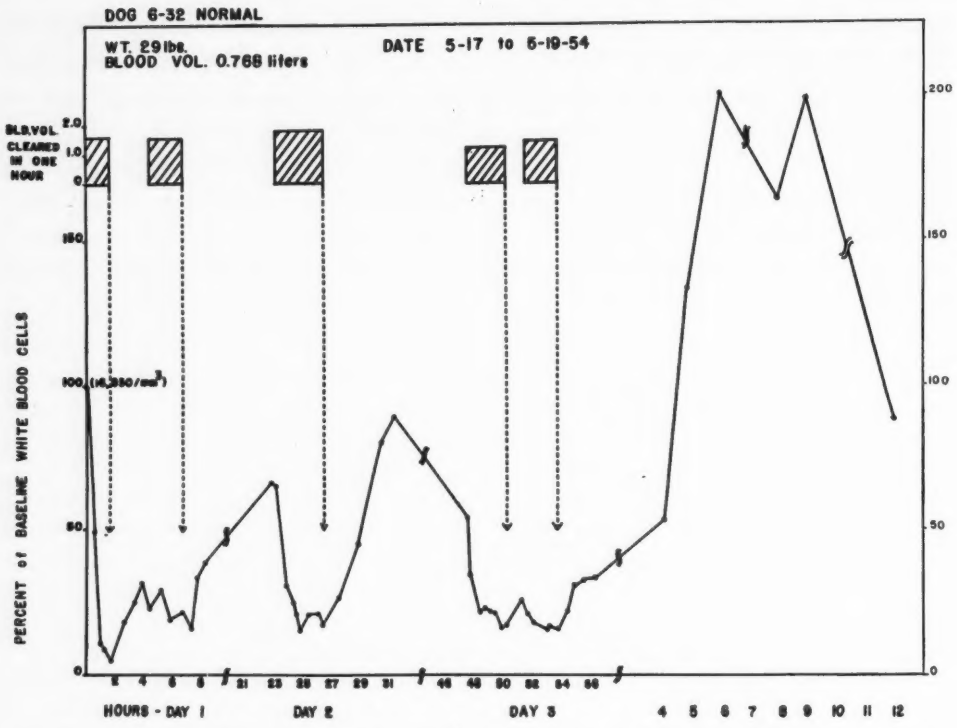

Fig. 10.—Alterations in leukocyte response of a normal dog to leukopheresis on 3 successive days. Here leukopheresis was repeated on the first and third days in an attempt to remove the maximum number of cells.

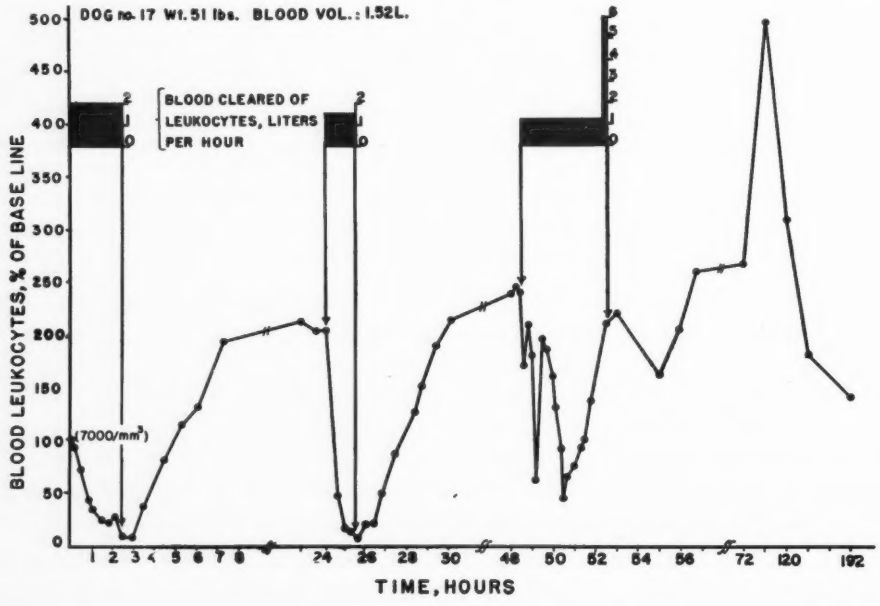

Fig. 11.—Alterations in leukocytic response of a normal dog to leukopheresis on 3 successive days. Note that the rise in blood leukocytes on the third day began while leukopheresis was under way at a very rapid rate.

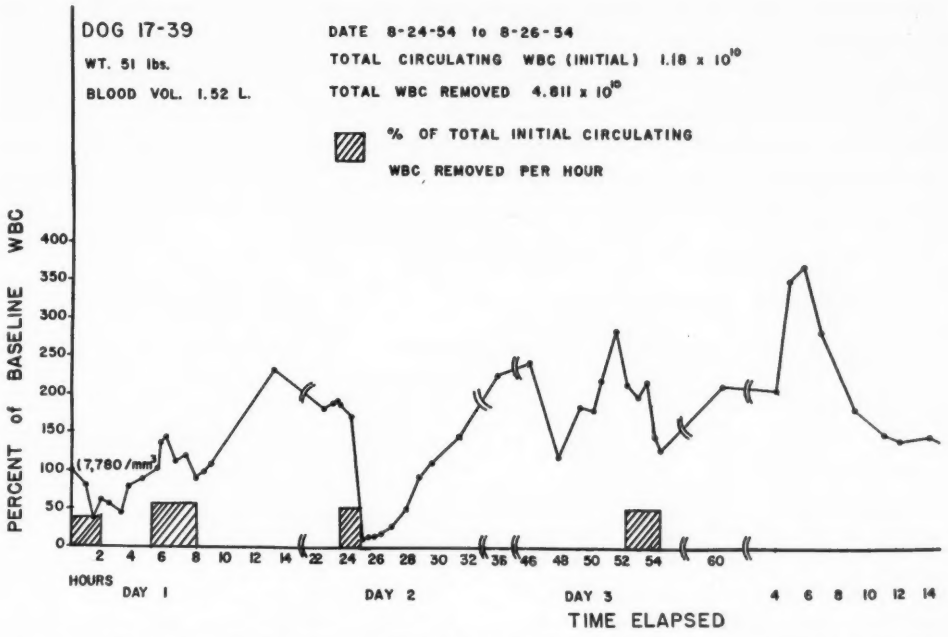

Fig. 12.—The leukocyte response in the same animal depicted in Fig. 11 after administration of 1 Gm. chloramphenicol for 40 days. Note that the rate of leukopheresis is expressed here as the percentage of circulating cells removed.

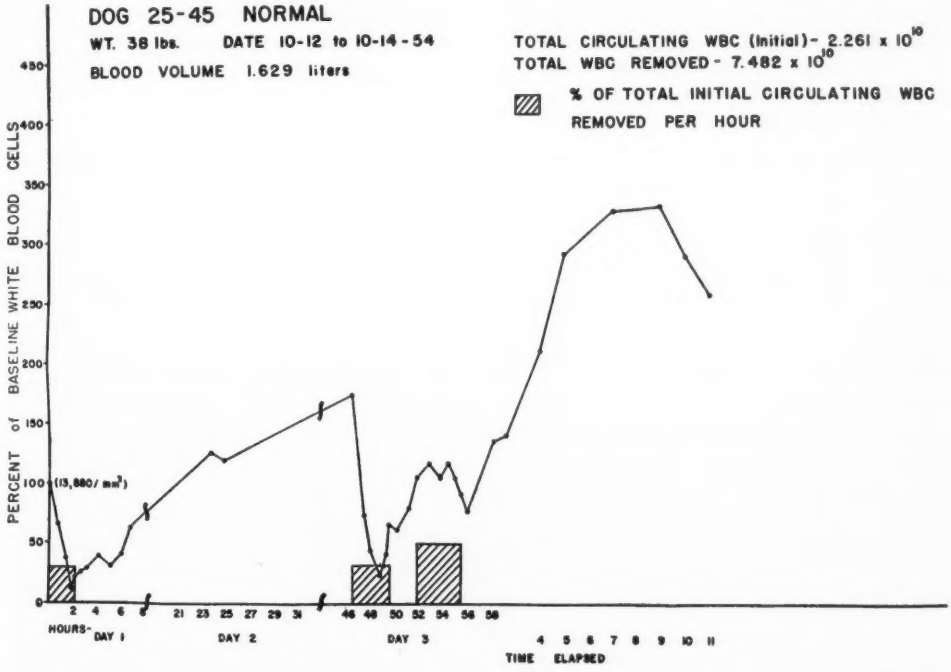
DOG 25-45 NORMAL
WT. 38 lbs. DATE 10-12 to 10-14-54
BLOOD VOLUME 1.629 liters
TOTAL CIRCULATING WBC (Initial)- 2.261 x 10^10
TOTAL WBC REMOVED - 7.482 x 10^10
% OF TOTAL INITIAL CIRCULATING WBC REMOVED PER HOUR
PERCENT of BASELINE WHITE BLOOD CELLS
(13,880/mm³)
HOURS
DAY 1
DAY 2
DAY 3
TIME ELAPSED

Fig. 13.—The leukocyte response of a normal dog leukopheresed on the first and third days. Note the removal of large numbers of cells during the last leukopheresis without production of leukopenia.

show the response in 2 normal dogs. Fig. 12 shows a further experiment on the same dog depicted in Fig. 11 after 38 days of chloramphenicol..* Fig. 13 shows the results in a normal dog leukopheresed on the first and third days only.

The general form of the response of these animals to repeated massive leukopheresis is similar. As would be expected, there are variations in the magnitude of response from animal to animal. The response to the initial leukopheresis

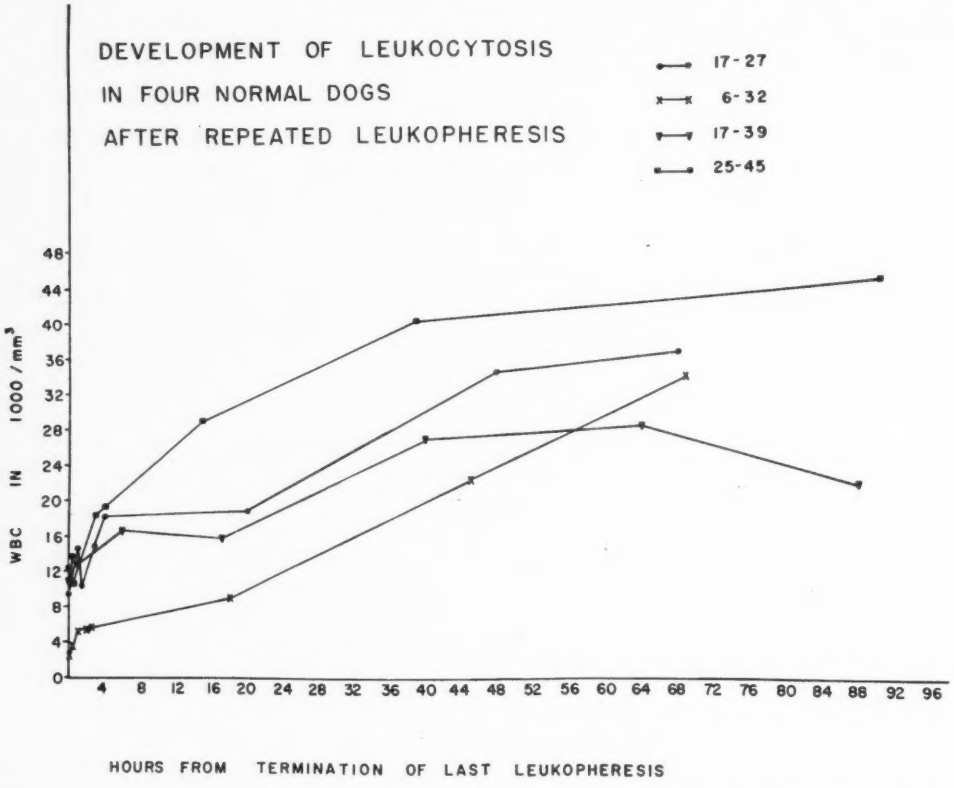

Fig. 14.—The delayed leukocytosis in 4 normal dogs after repeated leukopheresis. Values expressed in WBC per millimeter³.

was, of course, identical to that described previously. The recovery from leukopenia induced on the second or third day was very rapid. Recovery began as soon as leukopheresis was terminated, and the rate of recovery was more rapid than after the initial leukopheresis. In one instance (Dog 17-27, Fig. 11) the leukocyte level rose while leukopheresis was under way. In each instance large numbers of cells were removed, amounting to 3.24×10^{10} in Dog 17-27, 7.32×10^{10} in Dog 6-32, 4.81×10^{10} in Dog 17-39, and 7.48×10^{10} in Dog 25-45. It will be noted that each dog not only was able to replenish his peripheral blood with leukocytes but subsequently developed a marked leukocytosis. In terms of the demand imposed on the body supplies of leukocytes, the number removed must

*Results in regard to the effect of chloramphenicol on leukopoiesis will be the subject of a future report. This experiment is included here as a normal response.

be added to the number entering the circulation in response to the procedure. In only one dog (Fig. 10) did the imposed demand appear to exceed the supply during the 3-day period. It will be noted that in this animal leukocytes poured into the peripheral blood at an extremely rapid rate during and after the third leukopheresis. However, unlike the other animals, which rapidly attained a level of 100 to 200 per cent of base line, this animal reached a peak of about 60 per cent (or 10,000 per cubic millimeter) during the 18 hours following the final leukopheresis.

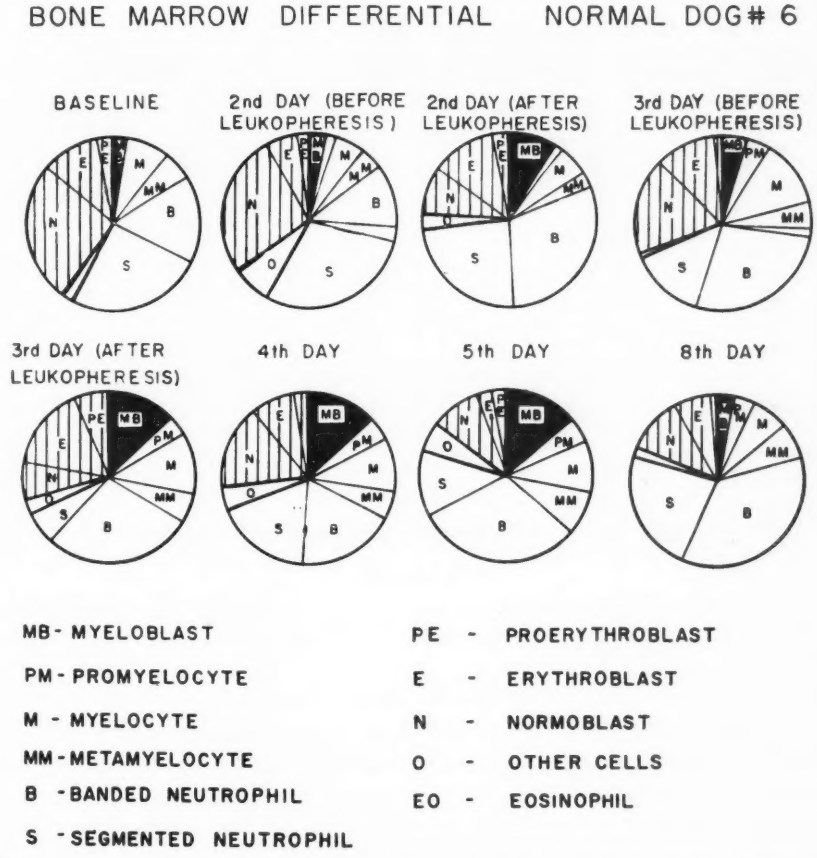

Fig. 15.—Demonstrating the gradual development of myeloid hyperplasia and immaturity becoming quite definite by the third day and reaching a maximum on the fifth day. This dog's blood leukocyte response is shown in Fig. 10.

Following the abrupt replenishment of the peripheral blood leukocyte count after the final leukopheresis, each animal maintained his count at this level for some 24 hours. Subsequently there was a gradual increment in white blood cell count, reaching a peak in about 40 to 70 hours after termination of the procedure. It is noteworthy that the slope of the curve for development of this leukocytosis after the recovery from the third leukopheresis is similar for each dog, as shown in Fig. 14.

The results obtained in these animals become more meaningful when correlated with serial marrow and blood differential counts. Although estimates of total cellularity are extremely crude by the methods employed, there did not seem to be any consistent over-all change in cellularity during the 3 days of leukopheresis. It was our impression that the cellularity was somewhat reduced immediately after each leukopheresis, but possible technical errors do not permit

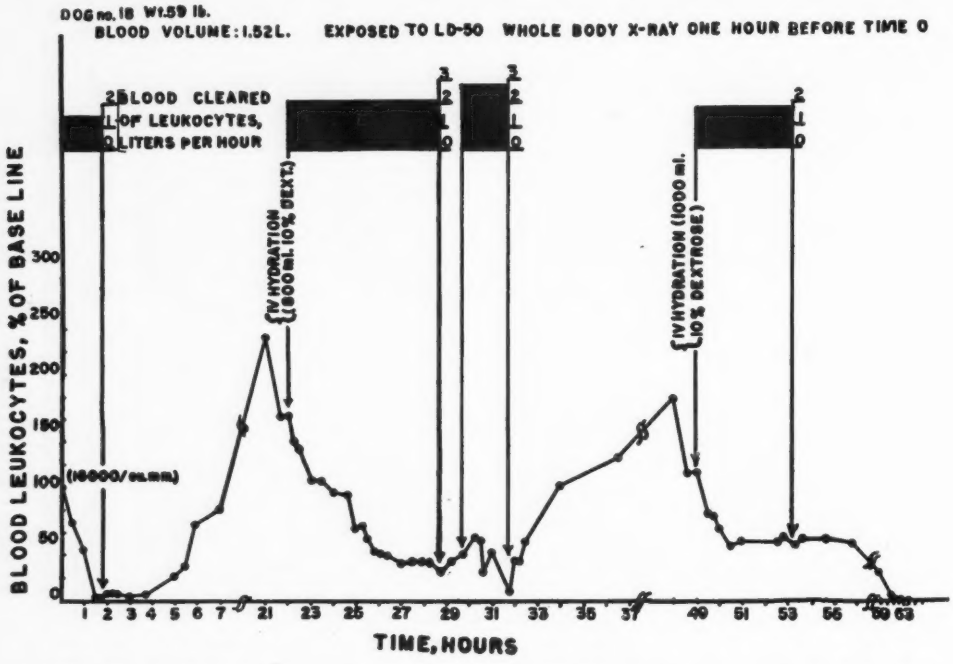
DOG no. 18 Wt. 59 lb.
BLOOD VOLUME: 1.52 L.
EXPOSED TO LD-50 WHOLE BODY X-RAY ONE HOUR BEFORE TIME 0
BLOOD CLEARED OF LEUKOCYTES, LITERS PER HOUR
IV HYDRATION (1000 ml. 10% DEXT.)
IV HYDRATION (1000 ml. 10% DEXTROSE)
BLOOD LEUKOCYTES, % OF BASE LINE
(16000/cu.mm.)
TIME, HOURS

Fig. 16.—Leukocyte response to repeated leukopheresis in an animal exposed to L.D.$_{50}$ whole body irradiation 1 hour prior to the first leukopheresis. During the second day's clearance, 15×10^{10} cells were removed. In all, 18.4×10^{10} cells were removed.

a definite statement. During the three days of leukopheresis there was no consistent change in the M/E ratio. However, immaturity of the myeloid series, apparent by the third day, became progressively more marked, reaching a maximum on the fourth or fifth day (1 or 2 days after the third leukopheresis) with marked myeloid preponderance. The data in one animal are depicted in Fig. 15.

The peripheral blood differential counts showed a moderate shift to the left beginning after the first leukopheresis and persisting or increasing with repeated leukopheresis. It is notable, however, that marked immaturity of the white cell population in the peripheral blood, as judged by morphology, was not encountered.

These data leave no doubt that the normal animal repeatedly rendered leukopenic by this means is capable of releasing relatively tremendous numbers of cells into the peripheral blood. The rate of release of these cells increases with demand and is associated with hyperplasia of the myeloid tissue in the marrow.

Irradiated animals: Figs. 16 and 17 indicate the results in two dogs exposed to L.D.$_{50}$ whole body x-ray 1 hour prior to leukopheresis. A third animal exhibited a similar picture.

Noteworthy is the fact that these dogs, each with a high base-line leukocyte level, behaved similarly to the previously described irradiated dogs after the initial leukocyte depletion. Thus, there was a prolonged delay after cessation of exchange before the leukocyte level began to rise, followed by a steady increase in the leukocyte count reaching a maximum at 24 hours. During this period of leukopheresis only 0.42×10^{10} cells were removed from Dog 18 (Fig. 16),

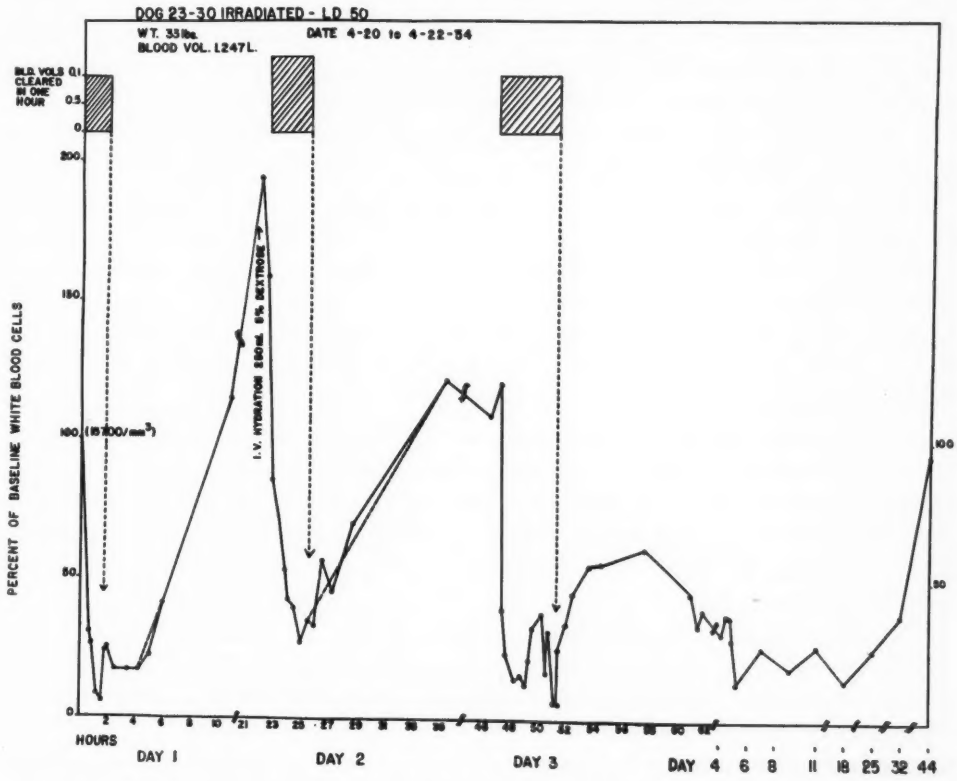
DOG 23-30 IRRADIATED - LD 50
WT. 33lbs.
BLOOD VOL. 1247L.
DATE 4-20 to 4-22-54
BLD. VOLS CLEARED IN ONE HOUR
PERCENT OF BASELINE WHITE BLOOD CELLS
I.V. HYDRATION 250ml. 5% DEXTROSE
HOURS
DAY 1
DAY 2
DAY 3
DAY 4 6 8 11 18 25 32 44

Fig. 17.—Leukocyte response to repeated leukopheresis in a dog exposed to L.D.$_{50}$ whole body irradiation 1 hour prior to the initial leukopheresis. This animal eventually recovered.

representing one-sixth of the total circulating cells. The second depletion was instituted at the 24-hour high point. Leukopenia was induced gradually and only after persistent depletion. It was obvious that leukocytes were entering the circulation at a greatly increased rate. During this prolonged exchange the cells removed by actual count amounted to 15.4×10^{10}. The number calculated to be in the circulation at the onset was 4.49×10^{10}. When leukopenia was finally attained after a short burst of extremely rapid turnover, an immediate linear rise in the count began when exchange was terminated. However, the height of the leukocytosis was not as great as after the first leukopheresis.

The third leukopheresis was instituted while the leukocyte count was quite high. Again it was evident that cells were entering the circulation at a surprising rate. The count was reduced to about 50 per cent of the base-line level, during which time 2.34×10^{10} cells were removed. The total number of cells removed over the 3-day period (by counts from the cell collection which are undoubtedly lower than the actual number removed) amounted to 18.2×10^{10} or some seven times the number originally circulating in the blood (4.49×10^{10}).

After the third leukopheresis the fecal discharge contained considerable numbers of myeloid cells. Rather than a return toward the base line, there was a slowly progressive fall in WBC, reaching extreme depths some 9 hours later. At this point the blood was virtually devoid of white cells. The animal expired about 3 hours later. However, the second animal survived and recovered completely. The third animal lived 72 hours after the last leukopheresis.

The marrow and peripheral blood morphology were entirely different from those in the nonirradiated dogs. At no point was immaturity of the cell population detectable, either in the marrow or peripheral blood. The marrow after the initial leukopheresis consisted almost entirely of stab and segmented forms, as did the peripheral blood. Each subsequent marrow showed essentially the same picture but with progressively fewer cells.

The final bone marrow smears on these irradiated dogs were essentially devoid of white cells in any form. The absence of cells was confirmed by marrow biopsy taken at autopsy of Dog 18.

The animal exposed to $L.D._{50}$ whole body x-ray and leukopheresed on 3 successive days which recovered completely showed the same general pattern of response of the myeloid tissue. Notable during the recovery period was the prolonged period of leukopenia despite evident regeneration and hyperplasia of the marrow which began 2 weeks prior to the rise in peripheral blood count to normal.

Autopsy of the animal which died was notable for the following reasons: (1) No significant hemorrhagic manifestations were apparent. (2) Considerable peripheral edema and pulmonary congestion were present as a result of the hypoproteinemia incident to gradual but cumulative loss of plasma proteins, plus an overtaxed myocardium. (3) In one area of the lung considerable inflammatory reaction was present as a result of hypostatic bronchopneumonia. Large numbers of mature myeloid cells were present in the lung parenchyma and alveoli at the area, but no intravascular leukocytes were seen. (4) The bowel revealed a chronic type of inflammatory change in the submucosa with very few myeloid forms visible. (5) The spleen was markedly contracted and showed no unusual histologic change. Polymorphonuclear leukocytes were strikingly absent from any part of the vascular tree. The marrow was aplastic in all respects.

DISCUSSION

It is clear that the normal dog has the capacity of supplying a greatly increased peripheral demand for leukocytes. Also, he can do so repeatedly without the development of an "exhaustion" picture. Indeed, in most instances the

blood was replenished with leukocytes more rapidly after repeated depletions. One aminal (Fig. 10) failed to reach as high a blood count during the period immediately following the third leukopheresis. However, the animal was by no means leukopenic and subsequently the white count rose to high levels. This was the only normal dog who showed any evidence of being unable to produce leukocytes at a sufficiently rapid rate to keep ahead of the removal of white cells.

The procedure of leukopheresis brings about an acceleration of the rate of entry of leukocytes into the peripheral blood. The initial leukopheresis invariably resulted in marked leukopenia if carried out at a rate of 1.5 blood volumes per hour for 3 hours. During this time about the same number of cells originally circulating were removed. This observation, together with the delay period after cessation of the procedure before the leukocyte level began to rise quickly, suggests that leukocytes enter the circulation at a relatively slow rate in the basal state. Following this delay period some change takes place, resulting in the rapid entry of white cells into the peripheral blood over the next 6 to 7 hours until a new level of circulating cells is reached. If leukopheresis is carried out during this period of developing leukocytosis it is very difficult to exceed the rate of entry of leukocytes. However, once the blood leukocyte count has reached its new level, which is generally in excess of base line, the rate of entry of cells into the circulation again slows. Leukopheresis on the following day, with the white count at this higher level, again results in leukopenia, although this is not so readily attained as initially. Recovery begins more quickly and reaches a higher level on the second day. On the third day leukopenia is induced with more difficulty and recovery sometimes begins while leukopheresis is under way.

These data indicate that massive removal of leukocytes from the peripheral blood results in leukopenia because the rate of release of these cells into the circulation is readily exceeded. However, after a certain delay the animal responds by accelerating the process of cell release. This process of leukocytosis continues for a few hours and then slows. Repeated leukopheresis after the leukocyte count has reached its maximum is accompanied by a progressive shortening of the delay period before leukocytosis begins.

The development of leukocytosis after leukopheresis does not depend entirely upon an intact marrow growth pattern. Those dogs subjected to heavy whole body radiation exhibited the same phenomenon after the initial leukopheresis although the delay before leukocytosis began was more prolonged. Leukopheresis on the second day was associated with the removal of large numbers of leukocytes and was followed by a progressively rising blood level, although this occurred more slowly and did not attain the same height as after the initial depletion. Again during the third day leukocytes were rapidly released into the peripheral blood until the stores of these cells became exhausted, as evidenced by marked depletion of the marrow. It is clear, therefore, that the removal of leukocytes from the circulation calls forth the accelerated release of white cells into the peripheral blood until there are no further cells to be released. The initial acute leukocytosis following leukopheresis must involve release of leukocytes from areas of storage primarily and acceleration of growth processes

secondarily. The acceleration in growth, which enables the normal animal to respond with a marked leukocytosis repeatedly, is probably a slow phenomenon. The most striking difference between the normal and irradiated animals is observed after the third day of leukopheresis.

The gradual increment in white cells over the 2 days following 3 successive days of leukopheresis, as seen in the normal dogs, may represent a different phenomenon from the immediate leukocytosis subsequent to depletion. The associated myeloid hyperplasia and the similarity of response of the various normal animals suggest that increased leukopoiesis is the predominant factor in this phase of the picture.

The ability of the heavily irradiated animal to develop a marked leukocytosis after leukopheresis indicates the large number of myeloid cells available for release into the peripheral blood, even though production of leukocytes by their earliest progenitors and mitosis have been seriously crippled or ablated. In Dog 18 (Fig. 16) some seven times the number of circulating cells (18.2×10^{10}) were removed. In addition many leukocytes were lost in the intestinal reaction to x-ray and other areas of inflammation. It is evident that the stores of leukocytes are sufficient to replace the number circulating in the peripheral blood many times.

A question which cannot be answered at this time is whether the stores of leukocytes contributing to the leukocytosis in the irradiated animals are entirely within the marrow. The marrow is the ultimate source of myeloid leukocytes in the blood and tissues (in the absence of myeloid metaplasia). If it is assumed that the majority of leukocytes removed during leukopheresis and those contributing to the subsequent leukocytosis were cells released directly into the peripheral blood from the marrow, then some conception of the great size of the marrow reservoir of myeloid cells is apparent.

However, the rapid increment in circulating leukocytes after leukopheresis could be contributed to by at least two other mechanisms:

1. Release of leukocytes from areas of *intravascular* sequestration such as the splenic and pulmonary vascular beds. The failure of intravenous epinephrine to alter the leukopenic state following the first leukopheresis is against this phenomenon being a major contributor. Furthermore, the progressive, linear rise in blood leukocytes does not suggest a sudden, massive influx of cells from such areas. Those animals subjected to more prolonged continuous leukopheresis in which leukopenia was maintained for several hours behaved similarly to those in which the depletion was terminated as soon as leukopenia was achieved. One would expect the former to have been depleted of sequestered cells and, therefore, exhibit an impaired recovery if this was a major source of cells. Further studies on splenectomized animals, for example, should help to establish the importance of this phenomenon to the over-all response to leukopheresis.

2. Re-entry of leukocytes from extravascular areas. The evidence pointing to a rapid turnover of leukocytes in the circulating blood has been discussed in the introductory remarks. Since the vascular system is not a closed circuit in so far as leukocytes are concerned, there may be many more white cells outside

the vascular tree than within. Osgood presented data to indicate an extravascular mass some 40 times that of the circulating leukocytes. It is possible that the procedure of leukopheresis promoted, in some way, the re-entry of cells from the tissues into the peripheral blood. Such a phenomenon, in order to continue, would require replacement of the leukocytes leaving the tissues by newly formed leukocytes. Therefore, regardless of whether re-entry of leukocytes from the tissues was a major source of leukocytes during the immediate post-leukopheresis response, the ability to develop a leukocytosis despite repeated depletions would require production of new cells by the marrow. Furthermore, it remains to be proved that leukocytes outside the vascular tree may re-enter the circulation in massive numbers.

The concept of there being large stores of relatively mature leukocytes in the marrow, and perhaps elsewhere, may be an important consideration in evaluating the effect of lesser degrees of marrow damage. Smaller amounts of irradiation, for example, could temporarily impair cell production without the development of significant leukopenia because the reserves of leukocytes are great enough to maintain the peripheral blood level until repair of the damage to cell production occurs. Depletion of the reserves by leukopheresis, however, should reveal growth impairment by an inadequate recovery from repeatedly induced leukopenia. Preliminary results employing smaller doses of irradiation indicate this to be the case.

The entry of leukocytes into the circulation becomes greatly increased after the massive removal of leukocytes from the peripheral blood. The removal of leukocytes must represent a stimulus to leukocytosis. How this stimulus is mediated is obscure. It is attractive to think that some humoral stimulus to leukopoiesis takes place, similar to that to erythropoiesis after red cell loss.[24, 25] Such indeed may be the case, but it seems probable that the leukocytosis involves release of preformed cells, at least during the period immediately following depletion of the peripheral blood. The evidence points to increased leukopoiesis occurring gradually, being necessary for the ability of the animal to respond with leukocytosis repeatedly but not to a single leukopheresis before the stores of myeloid cells have been depleted. Therefore, the stimulus of leukopheresis is twofold: a stimulus bringing about the release of preformed leukocytes and, if repeated, a stimulus to myeloid hyperplasia and accelerated leukopheresis.

Removal of leukocytes from the peripheral blood has not been considered among the causes of leukocytosis. However, when one considers that part of an inflammatory reaction depends upon myeloid cells leaving the circulation to localize at the site of tissue reaction, this phenomenon in itself represents removal of cells from the circulation. This removal may set in motion the release of leukocytes from areas of storage and the hyperplastic marrow changes associated with accelerated cell production as observed in our experiments.

The present experiments do not clarify the problem of leukocyte lifespan. They do indicate the ability of the normal animal to replace the numbers of cells circulating in the peripheral blood many times over in a short space of time. This ability involves the large numbers of myeloid cells within the hemopoietic

organ, and perhaps in other tissues, in relation to the number in the peripheral blood as well as acceleration in the rate of growth of myeloid tissue associated with hyperplasia. It is hoped that further studies of this type combined with isotopic methods of differentiating newly synthesized cells will yield more precise knowledge of the physiology of leukocytosis and leukopoiesis.

SUMMARY

A technique is presented for the experimental study of leukocyte production. This technique, termed "leukopheresis," involves the rapid removal of leukocytes from the peripheral blood without introduction of toxic agents which might impair marrow function.

The results of studies in normal and irradiated dogs indicate the following: Both the normal and irradiated ($L.D._{50}$ whole body) dog can rapidly replace the number of cells circulating in the peripheral blood many times. The normal animal responds to repeated leukopheresis by replenishing the peripheral blood at an increasingly rapid rate. The radiated animal shows more delayed replenishment and, if repeatedly leukopheresed, becomes unable to respond with leukocytosis as his stores of white cells become depleted. The ability to develop a leukocytosis depends, therefore, on adequate stores of leukocytes which can be rapidly released into the circulation. The ability to respond to repeated leukocyte depletions depends upon acceleration of production of myeloid cells which, in turn, maintains the supply of storage leukocytes which may be rapidly released into the peripheral blood. The evidence suggests that the so-called reserves of myeloid leukocytes are quite large.

The possible mechanisms of leukocytosis and the nature of the stimulus to leukocytosis in these experiments are discussed briefly.

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THE DYNAMICS OF PLATELET PRODUCTION AS STUDIED BY A DEPLETION TECHNIQUE IN NORMAL AND IRRADIATED DOGS

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AS PART of the procedure of leukopheresis previously described,[1] platelets were removed almost quantitatively from the peripheral blood. The response of the animal to this depletion afforded the opportunity to derive information as to its capacity for thrombocytopoiesis. The results obtained were remarkably uniform and indicate the restricted ability of the normal dog to supply platelets.

The findings are believed to have clinical as well as academic interest and point up the advisability of considering the limitations of thrombocytopoiesis in certain clinical situations.

In 1911 Duke[2] made observations on dogs depleted of platelets which were similar to those reported here. Duke was actually undertaking a study of fibrinogen synthesis rate and his data on platelets were incidental. His procedure essentially was to bleed the dog at frequent intervals, allow the blood to clot, and readminister the serum and red cells derived from the defibrinated blood. Platelets were removed by their utilization during clotting and incorporation into the fibrin clot. Duke observed marked thrombocytopenia which persisted in varying degree for three or four days.

The technique employed by us eliminates platelets from plasma by centrifugation and/or sedimentation of red cells by high molecular weight dextran. Blood removed from the animal in small but continuously withdrawn aliquots is cleared of leukocytes and platelets and readministered. This procedure, as previously described,[1] is well tolerated by the animal and results in no apparent injury. This permits study of the rate of entry of thrombocytes into the peripheral blood in the normal intact animal without the use of potentially toxic substances.

The results of such procedures in 34 dogs have led to the conclusion that thrombopoiesis displays certain unique dynamics which have been heretofore overlooked or unknown.

METHODS

The technique of leukopheresis and thrombocytopheresis has been described in detail.[1]

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Blood for platelet counts was drawn from the free-flowing blood of the femoral artery or abdominal aorta through polyethylene catheters into siliconed containers. In some instances multiple counts were taken simultaneously from venous blood (femoral vein) and capillary blood (ear puncture, in which case the usual direct platelet counting method was employed). In every instance of thrombocytopenia agreement within the limits of error of the counting methods was obtained in blood from these different areas. Base-line counts and counts made after recovery from the induced thrombocytopenia frequently yielded a slightly higher count in arterial than in venous blood (of the order of 25,000 per cubic millimeter).

Blood specimens drawn into balanced oxalate (Wintrobe) for red cell count, white count, hematocrit, and plasma protein determinations were obtained at the same time as the platelet count specimens. Blood smears were made directly from the fresh blood at appropriate intervals. The estimate of platelet numbers from examination of these blood smears agreed with the values obtained by counting.

The blood drawn into siliconed tubes for platelet counting was mixed with approximately one-half volume of Rees-Ecker fluid not containing the brilliant cresyl blue dye. After thorough mixing by hand for 5 minutes or after shaking a portion drawn into a red cell counting pipette for 5 minutes, smears were made from this blood. Excellent dispersion of platelets without clumping was obtained. Wright's stained smears were then examined, employing a Miller ocular disc to facilitate counting, and the number of platelets calculated from the red cell count performed on the oxalated sample drawn simultaneously, according to the method of Dameshek.[3] The method has the distinct advantages of (1) dispersion of platelets, (2) avoidance of counting artifacts by virtue of the characteristic staining and appearance of platelets, and (3) the availability of permanent preparations which permits recounting in doubtful cases.

Normal values in dogs by this method ranged from 189,000 per cubic millimeter to 395,00 per cubic millimeter with a mean of 280,000 ± 12,000. The accuracy of the method can be increased in instances of thrombocytopenia by counting several times the prescribed number of red cell areas. Normally the red cells in 25 to 30 ruled areas are counted. The technique of marrow aspirate preparation has been described.[1] Megakaryocyte quantitation from aspirated marrow is difficult and unreliable. We are inclined to agree with Berman, Axelrod, and Kumke,[4] who thoroughly evaluated the accuracy of available methods of megakaryocyte counting and concluded that the only correlation which could be made was, if megakaryocytes on a smear from aspirated bone marrow were very plentiful, then it was probable that the marrow contained an increased number of these cells. They showed the poor agreement of estimates from aspirated specimens with those made from marrow sections. For these reasons we have made no attempt to express the number of megakaryocytes in quantitative terms. However, when there was an obvious, marked increase in these giant cells on every slide prepared from a given aspirate (as shown in the photomicrographs) there would seem no doubt as to the plethora of these cells. We hope to be able to present more accurate data from marrow sections at a later date.

As part of the reactive pattern of the megakaryocytes to the induced thrombocytopenia, definite morphologic change in these cells with an increasing percentage of young forms was observed in the normal animals. Therefore, megakaryocytes were assessed and, on the basis of differential counts of 50 to 100 megakaryocytes for each marrow specimen, classified as megakaryoblasts, promegakaryocytes, or megakaryocytes. *Megakaryoblast:* Typically large cells but not so large as more mature forms and with only a thin rim of dark basophilic cytoplasm. The nucleus is dense, oval, or kidney-shaped, and with nucleoli infrequently visualized (probably due to the thickness of the cell). *Promegakaryocyte:* The nucleus still comprises most of the cell but it is frequently lobulated and less dense than the smaller, earlier cell. The reticular arrangement of the chromatin is visible. The cytoplasm is paler and irregular in outline but shows little evidence of platelet production. *Megakaryocyte:* The typical platelet-producing giant cell of the marrow. Nucleus

is markedly lobulated and variable in staining intensity. Cytoplasm is profuse and irregular in outline. Intracytoplasmic plateletlike structure can be seen with clumps of platelets around the cell. There are, of course, occasional arbitrarily classified gradations between these forms.

In the normal dog before leukopheresis many smears of marrow must be examined in order to visualize 100 megakaryocytes. The cells are usually found on the margins of the slide or the feather edge. The cells observed are almost entirely mature megakaryocytes or promegakaryocytes with abundant platelet production.

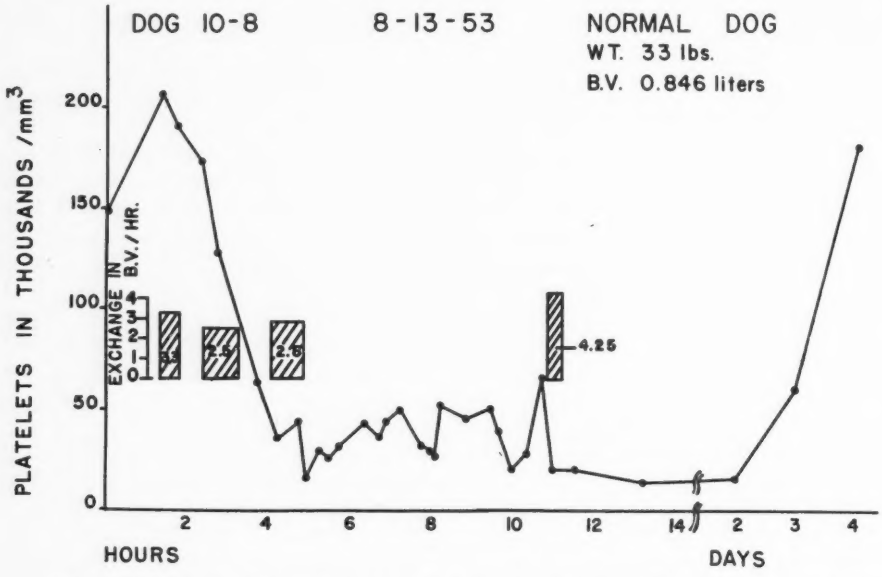


Fig. 1.—Response of blood platelets in a normal dog leukopheresed on 1 day only.

Six animals were subjected to whole body irradiation at a dosage of 250 r administered from two x-ray heads, one above and one below the animal, as described previously in detail.[1] This exposure represents the approximate 50 per cent lethal dose for dogs, and preceded the onset of the depletion procedure by 18, 36, and 42 hours in 3 dogs and 1 hour in the remaining 3. The elapsed time was much shorter than that required for the spontaneous development of thrombocytopenia following exposure to LD 50 whole body x-ray. As previously discussed,[1] none of these animals exhibited any symptoms of radiation sickness at the time leukopheresis was performed. All animals in both the normal and the x-ray groups were in good nutritional condition without subjective or objective evidence of infection. All had been observed for at least a 2-week period before their experimental use.

Penicillin and streptomycin were administered on the days of leukopheresis only. No evidence of wound infection developed. Blood cultures obtained before, during, and after the period of leukopheresis were negative in 6 animals.

RESULTS

The effect of leukopheresis on blood platelet levels with the subsequent return of platelets into the blood in two typical experiments are shown in Figs. 1 and 2. The platelet levels in the peripheral blood of 9 normal and 3 irradiated dogs (LD 50 whole body x-ray) subjected to a single period of leukocyte and platelet depletion are shown in Fig. 3. The points on the curves

represent average figures. The spread of base-line values was quite wide from animal to animal (±103,000) but the pattern of response was similar. A thrombocytopenia of 30,000 per cubic millimeter or lower was induced in every normal animal. Platelet counts were performed at frequent intervals by the method described so that values could be rechecked if necessary. The low platelet levels were established beyond doubt by serial repeated counts, examination of blood smears, and correlation with observations of clot retraction and prothrombin consumption (functions which are dependent upon adequate numbers of platelets).

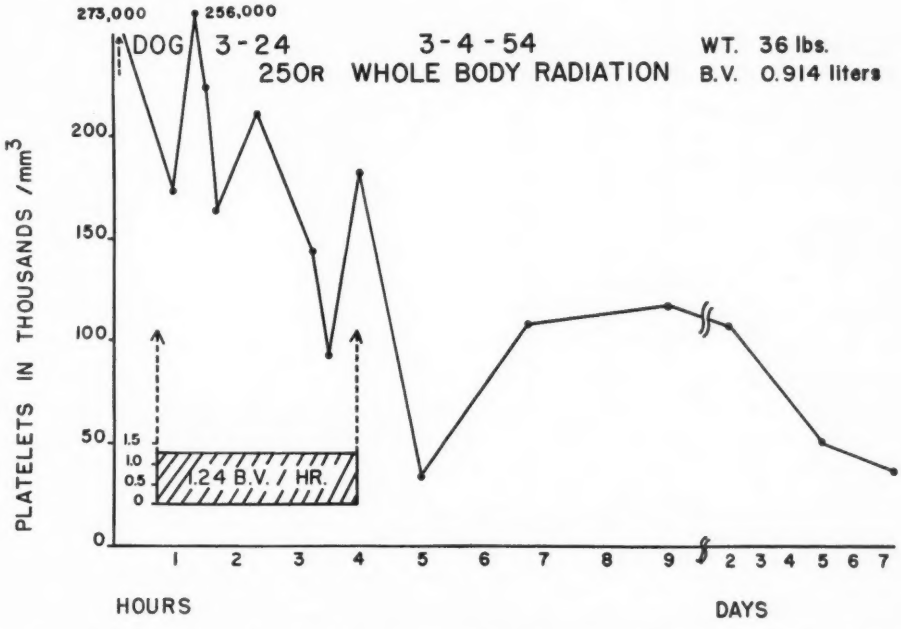


Fig. 2.—Response of blood platelets in an irradiated dog (L.D.$_{50}$ whole body) to a single leukopheresis.

The extent of platelet depression at the termination of leukopheresis depended upon the total amount of blood which had been cleared of platelets and was not as greatly affected by the speed of clearance as was the case with the leukocytes. In every animal the clearance of 2 to 3 blood volumes resulted in severe thrombocytopenia (0 to 10 per cent of base line). In animals subjected to lesser degrees of leukopheresis, milder thrombocytopenia ensued. It is probably for this reason that the platelet level was not reduced as severely in the irradiated dogs (Fig. 3) after the initial leukopheresis. The rate and duration of leukopheresis were controlled by the white blood cell count, and since this was much more rapidly lowered in the irradiated than in the normal dogs,[1] less blood was cleared of platelets in the former. There may, however, be other factors concerned in the differences in the degree of thrombocytopenia observed after the initial leukopheresis, such as an increased rate of cell maturation, as suggested by Bloom and Jacobson.[5]

It will be noted that the average recovery curve of blood platelets (Fig. 3) was diphasic in type. In most normal animals there was an immediate progressive rise in blood platelets after cessation of leukopheresis. This rise was suboptimal and transient. Furthermore, some normal dogs failed to show any rise of this type. The platelet count had fallen spontaneously by the following morning and remained depressed during the second and third days.

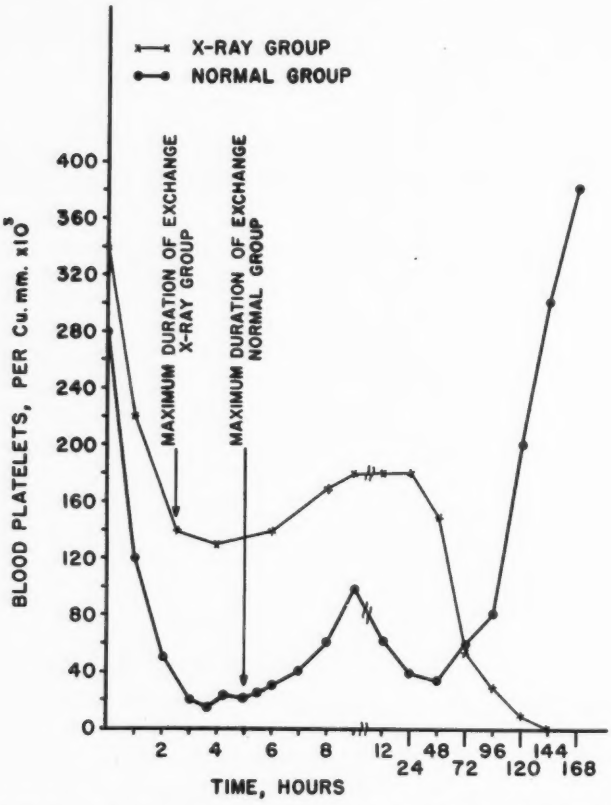


Fig. 3.—Response of blood platelets in 9 normal and 3 irradiated dogs to leukopheresis on 1 day only.

Subsequently there was a progressive rise, reaching normal on the sixth or seventh day after leukopheresis. The irradiated animals showed a progressive fall in platelets beginning at 48 hours after leukopheresis.

Serial tests of blood clotting* were performed in 10 animals. Clotting time was normal in all animals not receiving dextran except 2 which were given heparin inadvertently in the process of keeping the arterial cannula patent. In all animals receiving dextran incident to the use of this material to facilitate separation of leukocytes and platelets, prolonged clotting times were

*The method of the various clotting tests employed were as follows: Clotting time—Lee-White method. Prothrombin time—method of Ware and Strangnell.[6] Prothrombin consumption—method of Stefanini,[7] using bovine prothrombin-free plasma prepared by the method of Ware and Stragnell.[6] Bleeding time—a clean cut 2 mm. in depth and 3 mm. in length was made through the skin of the inner aspect of the ear. In most dogs bleeding occurs for only a few seconds. In instances of prolonged bleeding time, oozing continued for 10 minutes or more.

observed. Dogs with prolonged clotting times due either to dextran or heparin exhibited prolonged bleeding times and developed hemorrhagic complications, usually from the operative site or from the gastrointestinal tract. None of the other animals exhibited hemorrhage despite the thrombocytopenia. For this reason the data on platelet production were considered acceptable only in those dogs not receiving dextran or heparin.

Impairment of clot retraction and prothrombin consumption was observed in all animals rendered thrombocytopenic in which such tests were performed (10). These findings correlated with the degree of thrombocytopenia in those animals receiving dextran (4) as well as those receiving none (6).

Purpura was not observed in any animals. However, two dogs which had received dextran during the procedure and which died during the thrombocytopenic period showed widespread hemorrhages throughout most organs except the skin. Autopsy on 5 animals not receiving dextran or heparin which expired during the thrombocytopenic period revealed no evidence of hemorrhagic phenomena.

The results observed in 2 normal and 2 irradiated dogs subjected to repeated leukopheresis on 3 successive days are shown in Fig. 4. It will be noted the blood platelet numbers behaved in a manner roughly similar to those subjected to a single day's leukopheresis. Steady recovery in the 2 normal dogs began at the same time and followed the same curve as in the group exchanged only once. The similarity of the duration of thrombocytopenia in the animals leukopheresed for 3 successive days and those in which platelets were removed only once has significance with respect to the maturation time of megakaryocytes, as will be discussed subsequently.

Autopsy performed on one irradiated animal 9 hours following the last leukopheresis revealed no significant hemorrhages by gross or microscopic examination.

The marrow morphology was of great interest. During the course of the thrombocytopenia, megakaryocytic changes occurred and were quite helpful in evaluating the observed results. Appearance of increased numbers of megakaryocytes, particularly of the earlier types began to be noticeable by 24 hours after the initial depletion. This hyperplasia and immaturity became more marked during the ensuing 2 days, and by the fourth day was profound. Little or no "platelet production" could be seen in terms of platelets in or around these immature megakaryoblasts and promegakaryocytes. Indeed, large numbers of marrow platelets were not observed until the platelet counts in the peripheral blood had risen on the third or fourth day following leukopheresis. Following the return of blood platelets to normal marrow, aspirations were not performed at frequent intervals so that the duration of the megakaryocytic hyperplasia is not known in detail. However, from random observations in a few dogs it was apparent that the hyperplasia persisted for several days, during which time the platelet count was higher than before the procedure.

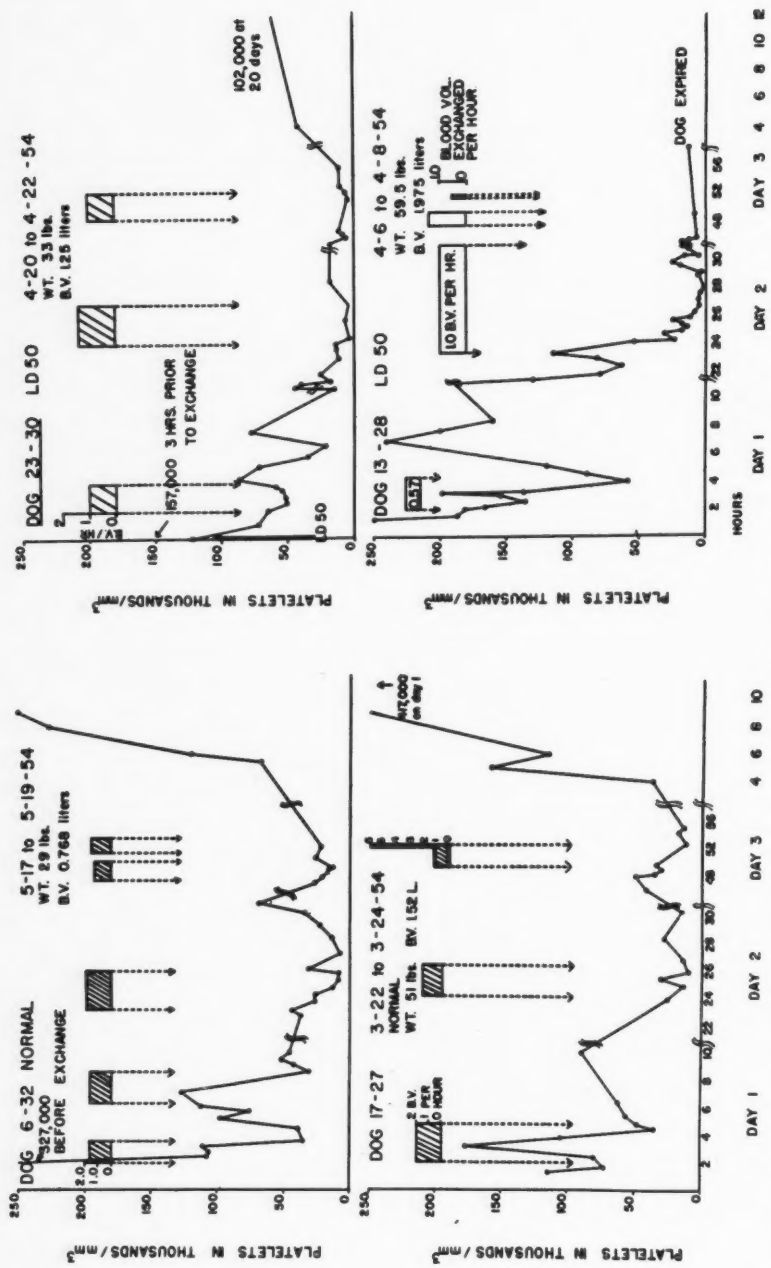
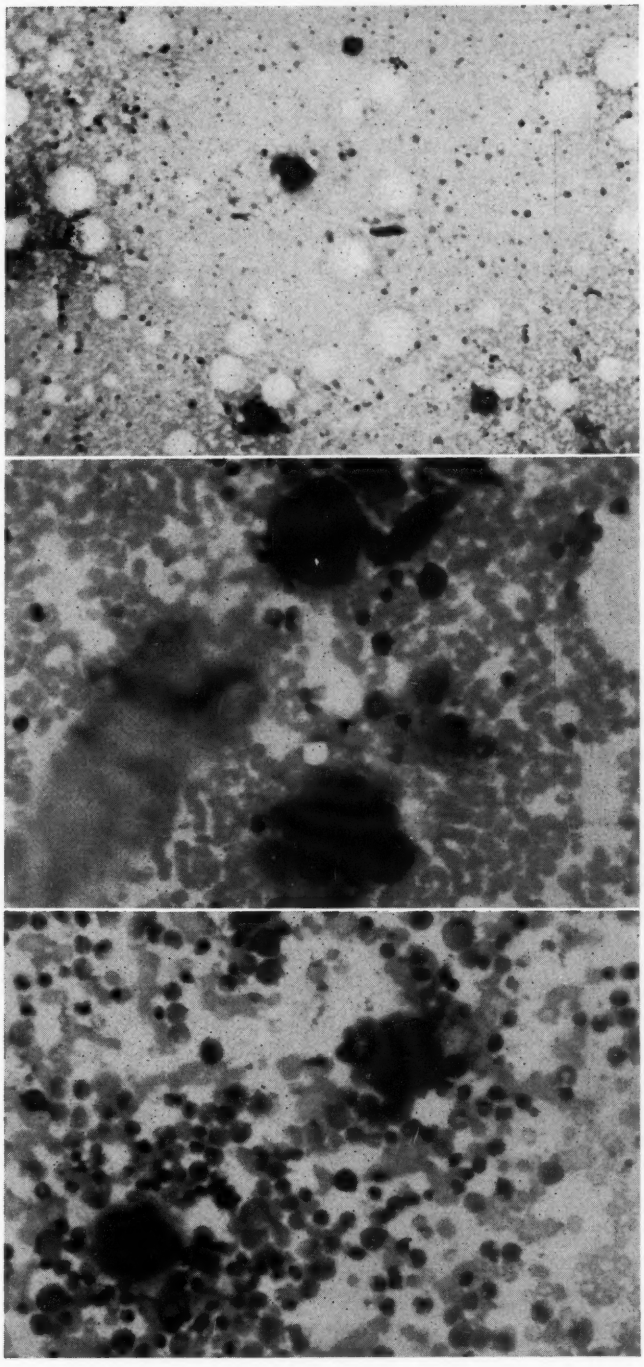


Fig. 4.—Effect of repeated leukopheresis for 3 successive days on blood platelets in 2 normal (left) and 2 irradiated (right) dogs.



A.

B.

C.

Fig. 5.—*A*, Low-power magnification of marrow of normal dog on day following 3 days of leukopheresis. Note the evident megakaryocytic increase.

B, High-power magnification of same marrow as in *A* showing the marked immaturity of some of these cells.

C, High-power magnification of normal dog's marrow 6 days after third leukopheresis. Megakaryocytes hyperplastic but more mature.

The marrow findings in those animals subjected to repeated leukopheresis did not differ essentially from those described, with respect to megakaryocytes.

A, B, and *C* in Fig. 5 are photomicrographs showing the type of megakaryocytic hyperplasia observed.

DISCUSSION

The consistency of the pattern of delayed recovery of blood platelet levels after mechanical removal of these elements from the peripheral blood indicates that in the normal animal the supply of platelets available for release into the peripheral blood is limited. The ability of the marrow to replace platelets may exceed the normal rate of utilization of platelets by a considerable margin. However, when the removal of platelets is massive, as in our experiments, this ability of the normal marrow is readily exceeded.

It is apparent that once thrombocytopenia has been produced in the normal animal by removing platelets at a rate exceeding that of entry of platelets, the blood remains deficient in platelets for 3 to 4 days, despite megakaryocytic hyperplasia in the marrow. The most reasonable interpretation of this apparent paradox is that the immature cell is not capable of producing platelets at a rate sufficient to elevate the levels in the peripheral blood. It would appear that in these dogs the response of the marrow was to accelerate the differentiation of immature megakaryocytes to replace those mature and degenerating megakaryocytes whose platelet-producing potential had been exhausted by the increased demand for platelets. However, such earlier forms require some 3 days to reach the point of maturity where they are capable of shedding these elements in large numbers. Such an hypothesis is supported by the morphologic evidence of sparse numbers of platelets in and around these more immature forms and by the progressive recovery of blood platelet levels to normal after 3 to 4 days.

It must be emphasized, however, that the lack of visible platelet formation in and around megakaryocytes does not necessarily denote an absence of platelet production. It is entirely possible that large numbers of platelets are being elaborated and their release into the peripheral blood is so accelerated that they are not seen in the marrow. If such were the case one would expect to find a more adequate maintenance of the blood platelet level, unless the peripheral utilization of these elements were so accelerated as to exceed the increased production. This possibility cannot be entirely eliminated as an explanation of the observed results. Certainly in those instances where hemorrhagic complications developed, peripheral utilization was increased. Therefore, the data in such animals were not considered valid. However, in those animals exhibiting no clinical evidence of bleeding (confirmed by autopsy in some instances) the likelihood of increased peripheral utilization of platelets as the main cause of the prolonged thrombocytopenia seems remote. The consistency of the platelet depression and the response in those

animals leukopheresed for 3 successive days support the interpretation of decreased platelet production by immature megakaryocytes as the most probable explanation.

The similarity of duration of thrombocytopenia of normal dogs leukopheresed on 3 successive days to those undergoing a single depletion procedure deserves comment. It is obvious that the repeated leukophereses were performed during the interval when thrombocytopenia existed incident to the initial leukopheresis. During this time platelet production was presumably inadequate due to immaturity of the megakaryocyte population in the marrow. Therefore, no further significant depletion or marrow alteration was incurred as a result of the second and third periods of leukopheresis. These data also indicate that the procedure of leukopheresis per se imposed no inhibitory effect on platelet production, since, if this were the case, recovery would have been further delayed by the repeated leukophereses.

The question of whether the megakaryocytic increase in the marrow, once the population of these cells has reached maturity, results in an increased rate of production of platelets is raised by the finding of blood platelet levels higher than base line after recovery from the thrombocytopenic state. One experiment in which a normal animal was leukopheresed on the sixth day, at which time the circulating platelets had been restored, failed to develop thrombocytopenia. The observations agree with those of Crosby and Howard[8] in battle casualties in the armed forces. These workers reported no evidence of thrombocytopenia in wounded men who had lost large quantities of blood and received massive replacement with citrated bank blood known to be low in platelets. The suggestion was made that blood loss prior to the time of transfusion may have accelerated platelet production. If our results can be compared to platelet production in human beings, no other explanation for the failure to observe thrombocytopenia in such instances is readily apparent. The fundamental effect of our depletion procedure is similar in most respects to the situation of massive blood loss and replacement of red cells and plasma. That citrated blood stored in glass contains virtually no intact platelets is well documented.

The initial increment in blood platelet levels subsequent to their depletion by removal is probably the resultant of several factors. Entry of platelets into the systemic circulation from splenic and pulmonary areas of sequestration is a definite possibility. Also the possibility exists that this temporary increase is contributed to by those megakaryocytes remaining in the marrow still capable of releasing platelets before their potential is finally expanded. Data on this point, as for example in splenectomized dogs, have not been obtained to an extent permitting conclusions.

The results in irradiated animals would appear entirely compatible with the necessity for replacement of "exhausted" megakaryocytes by succeeding generations in order to replenish the animals' blood with platelets. As in the case of production and release of myeloid leukocytes,[1] the irradiated animal responded by the rapid release of platelets and maintained the peripheral blood level at least as well as the normal dog until the existing stores of plate-

lets were depleted. Once this occurred, marked thrombocytopenia developed. The marrow reserves of platelets would seem to be less adequate than those of myeloid leukocytes in so far as replenishing the peripheral blood after repeated leukopheresis is concerned.

All the animals rendered thrombocytopenic which were tested demonstrated the defects in tests of coagulation expected in this situation (i.e., impaired clot retraction and prothrombin consumption). None showed hemorrhagic manifestations clinically or pathologically unless dextran or heparin had been administered. In such instances varying degrees of abnormal bleeding were observed and this was associated with prolonged clotting time and prolonged bleeding time in addition to the previously mentioned abnormalities. These observations are compatible with those of many others that thrombocytopenia per se is not necessarily accompanied by abnormal bleeding (e.g., purpura) unless some other abnormality is present. Ledingham,[9] Ledingham and Bedson,[10] and Bedson[11] emphasized this important point in their early studies of experimental thrombocytopenia. Purpura was invariably encountered in animals rendered thrombocytopenic by antiplatelet serum. However, if the platelet count was lowered to comparable depths by injection of agar intravenously, no purpura was observed. If these latter animals underwent an induced antigen-antibody reaction (e.g., hemolysis due to injection of an antired cell serum), purpura developed. Bedson concluded that a combination of vascular disturbance and thrombocytopenia was necessary for the development of purpura. This concept has been supported by many more recent reports. Many workers have shown that the combination of thrombocytopenia and some other coagulation defect (e.g., the anticoagulant effect of heparin) is more likely to result in spontaneous hemorrhage than either defect alone.[12]

The abnormal bleeding evidenced by those dogs rendered thrombocytopenic and receiving dextran indicates that dextran in high concentration exerts an anticoagulant effect which can become serious in the face of thrombocytopenia. This is presumably the same phenomenon as the observed increased sensitivity to the anticoagulant effect of heparin if thrombocytopenia is present.[12] Polysaccharides in general exhibit some degree of anticoagulation when present in high concentrations.[13, 14]

It is of note that those dogs subjected to repeated leukophereses after exposure to LD 50 whole body x-ray failed to manifest purpura. Autopsy in 1 of these dogs 9 hours after the third leukopheresis failed to show any evidence of hemorrhage. These findings may be of significance in evaluating the hemorrhagic manifestations of the postirradiation syndrome.[15, 16]

The relevance of these experimental results to clinical situations is indicated by recent reports of thrombocytopenia after acute blood loss. Desforges and associates[17] described the consistent depression of blood platelets in patients undergoing massive gastrointestinal hemorrhage with replacement transfusion. Although the thrombocytopenia was mild in most cases and deemed of little clinical significance, every case studied showed some degree

of thrombocytopenia. These workers were unable to find any cause for the observed platelet depression in terms of circulating toxins or antibodies. It is presumed, from the data presented herein, that the platelet deficiency was on the basis of inadequacy of the normal marrow to supply the demand for platelets imposed by the acute loss of blood, and replacement with platelet-free blood.

The degree of platelet depression incident to blood loss and replacement transfusion depends upon the quantities involved. In situations where exchange of blood in human beings approaches the volume of blood cleared of platelets in our experiments (i.e., 2 to 3 blood volumes in the course of 1 to 2 hours), severe thrombocytopenia would be anticipated. Such situations might develop in exchange transfusion therapy for hemolytic disease of the newborn infant, extracorporeal pumps as used in cardiac surgery, massive hemorrhage, and replacement as in certain types of vascular surgery. Studies are under way in such situations to determine the degree of thrombocytopenia encountered. Two cases of massive bleeding and transfusion in association with surgery for abdominal aneurysms demonstrated severe thrombocytopenia and continuous oozing at the operative site. These cases indicate the importance of this type of thrombocytopenia and will be included in a future report on this subject.

Desforges and associates[17] observed that the thrombocytopenia in their patients persisted for 3 to 4 days after cessation of bleeding. This period of thrombocytopenia corresponds to that observed in our animals. It is probable that the same process of immaturity of the hyperplastic megakaryocytes was associated with the prolonged period of thrombocytopenia. This aspect of the problem, i.e., the duration of thrombocytopenia after acute removal of platelets, emphasizes the possibility of hemorrhagic complications during this period.

The possible importance of posthemorrhage, posttransfusion thrombocytopenia demands further study. It may prove sufficient to warrant reconsideration of transfusion methods in some situations. If it is true that thrombopoiesis remains inadequate for several days after thrombocytopenia is induced by such events, replacement of platelets would seem feasible. This can be accomplished by direct transfusion,[18] the use of the multiple siliconed syringe technique,[19] or collection of blood in nonwettable systems.[20, 21] The practicality of separation of platelets on a large scale from human donors and the preservation of such platelets for subsequent intravenous use is suggested by the work of Tullis.[21] More detailed knowledge of the real significance of platelet antigenic differences is needed to clarify the feasibility of platelet transfusion.

The implications of these experiments with respect to the present concept of idiopathic thrombocytopenic purpura (I.T.P.) require comment. The changes brought about in the morphology of megakaryocytes by increasing the demand for platelet production by removal of platelets resemble to some extent those described by Dameshek and Miller[22] and others[23] as characteristic of I.T.P. It is evident that some of these changes are due to the im-

maturity of the hyperplastic marrow attempting to compensate for the increased rate of peripheral removal of platelets. In I.T.P. the removal is thought to be the result of platelet destruction by circulating toxins (probably antibodies).[24] Whether or not there is some immune[25] or humoral agent (as from the spleen) which inhibits platelet production by the megakaryocytes has been long debated.[26] That the megakaryocytes are not functionally damaged by a circulating antibody in I.T.P. would seem evident from the thrombocytosis occurring after splenectomy, despite the continued presence of the platelet antibody.[25] Whether or not the spleen actually suppresses thrombopoiesis by humoral means in this condition cannot be determined from the available literature. The results obtained in normal dogs rendered thrombopenic by removal of platelets, in which there was no reason to suspect "toxic" suppression of thrombopoiesis, may help to clarify this controversy. In our experiments it is clear that the massive removal of platelets resulted in thrombopenia which persisted until maturation of younger megakaryocytes occurred. In the face of peripheral removal of platelets by lysis, as in I.T.P., this initial inadequacy of platelet production would abet the thrombopenic state. Recovery from this thrombopenic level would not occur unless the rate of production exceeded the rate of peripheral destruction. If this destruction were continuous, platelet production might be inadequate for long periods of time.

SUMMARY

1. Thrombopoiesis in normal and irradiated dogs has been studied by removal of platelets from the peripheral blood at a rate exceeding their entry from the marrow.

2. Several consistent findings of significance were observed:

(a) Severe thrombocytopenia can be produced in the normal dog by merely removing platelets.

(b) The resulting thrombocytopenia persists, after a brief and limited remission, for 3 to 4 days in normal animals. Subsequent to this there is a progressive thrombocytosis in normal dogs, but thrombocytopenia persists in the irradiated dogs (exposed to LD 50 whole body x-ray).

(c) The megakaryocytes in the marrow of normal dogs become increasingly plentiful and immature in appearance during the early phases of the induced thrombocytopenia.

(d) Repeated platelet depletions on 3 successive days do not alter the response of the animal, platelets returning to the blood at the same time as in dogs rendered thrombopenic only once.

3. The concept is presented that the prolonged thrombocytopenia after a single depletion procedure is due to inadequate platelet production by immature megakaryocytic cells.

4. The possible relevance of the observed dynamics of the thrombopoiesis in these animals to various clinical conditions is discussed.

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ERYTHROCYTE GLYCOLYSIS IN HEMOLYTIC DISEASE

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IT HAS been known for many years that whole blood in vitro utilizes glucose and that most of the glycolytic activity is due to erythrocyte metabolism.[1] Almost all of the energy requirement of mature human erythrocytes is derived from glucose degradation, and only minimal oxygen consumption by mature red blood cells has been detected.[2] Several investigators have noted that blood containing large numbers of reticulocytes exhibits increased glucose utilization as well as demonstrable oxidative metabolism.[2, 3] The present study is concerned with the glycolytic rates of erythrocytes obtained from subjects with hemolytic disease and other diseases associated with reticulocytosis. It was undertaken as an investigation of the relationship of reticulocytosis and maturity of red cells to the glycolytic activity of blood. It was also felt that such a study might detect some gross metabolic defect in the erythrocytes of patients with hereditary hemolytic syndromes.

MATERIALS AND METHODS

Venous blood was collected with a small amount of dissolved heparin as an anticoagulant. The sample was spun for 10 minutes in a small-angle centrifuge at approximately 2,000 rpm, the plasma was aspirated into a separate tube, and the buffy layer removed. The packed red cells were then suspended in an equal volume of the aspirated plasma. Blood prepared in this way was found to have a white cell count below 3,000 per cu. mm., and most of the platelets were removed. The reticulocyte count was always in close agreement with that of the unaltered blood.

The reconstituted specimen was then divided. A hematocrit was done on one portion, glucose determination on another, and 1 ml. was placed in a 50 ml. Erlenmeyer flask or Warburg vessel. This sample was incubated in either a serologic water bath with shakers or a Warburg bath. Temperature of the baths varied from 37.2° to 38.0° C., and rate of agitation varied from 100 to 116 cycles per minute. No differences in results were noted between the two baths. No demonstrable hemolysis occurred during the period of incubation.

Glucose determinations by the Somogyi method[4] were done before and after the incubation period of 1 to 2 hours, and glycolytic rates were calculated on the basis of hematocrit and expressed as milligrams of glucose utilized per 100 ml. of packed cells per hour. Longer periods of incubation result in total disappearance of glucose from samples which exhibit active glycolysis, and we felt that the short incubation time was preferable to artificial elevation of the substrate by addition of glucose.

Patients with hematologic diseases were studied completely in our laboratory and diagnoses were established by appropriate tests. Reticulocytes were counted by the method of Brecher.[5]

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RESULTS

Control Studies.—Erythrocyte glycolysis varied considerably in a control group consisting of normal subjects and patients with miscellaneous diseases (hypothyroidism, hyperthyroidism, panhypopituitarism, diabetes mellitus, lupus erythematosus, rheumatoid arthritis, and Hodgkin's disease). The mean glycolytic rate established by our method in this group of 28 subjects was 43 mg. per 100 ml. of packed red cells per hour, with a standard deviation of ±8.8 mg. These values are somewhat higher than those obtained by Bartlett and Marlow[6] using a similar technique.

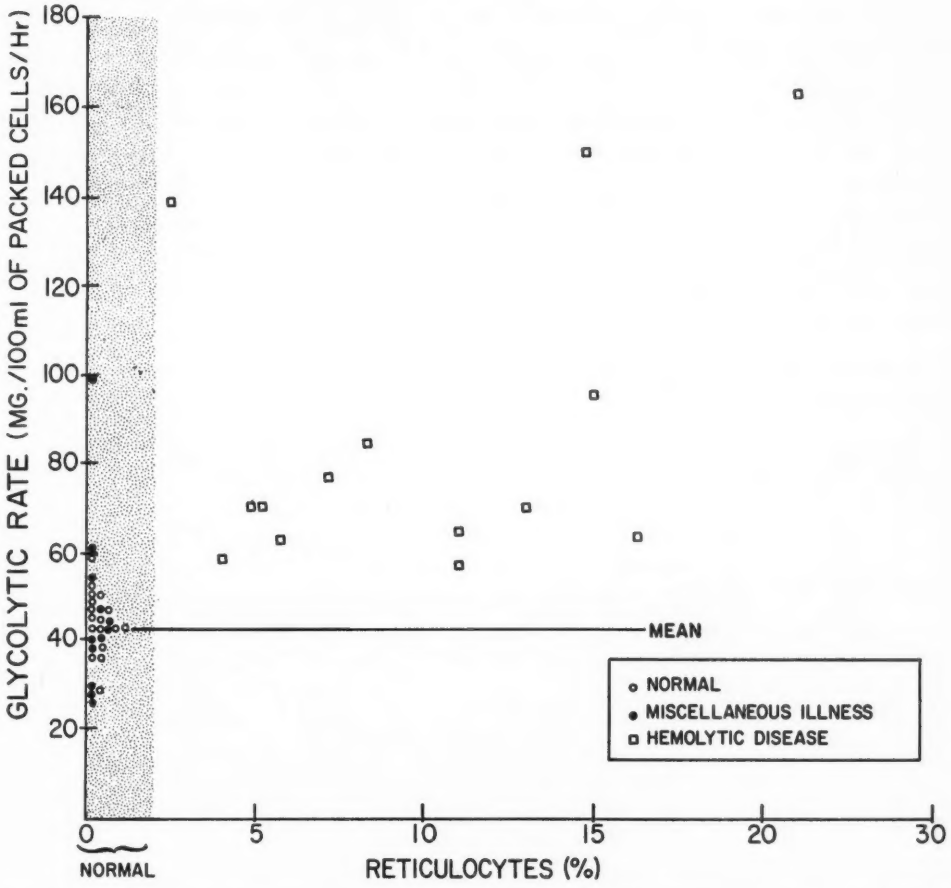

Fig. 1.—Relationship between erythrocyte glycolytic rate and reticulocyte count in control subjects and patients with hemolytic disease.

One subject in the patient group, included in the chart shown in Fig. 1 but not included in the calculation of the mean, had a glycolytic rate of 98 mg. —much higher than any other in the group. Unfortunately, the patient left the hospital before a second determination could be performed. The patient had complained of episodes of morning weakness, and had been sent to Walter Reed Army Hospital for evaluation of postprandial hyperglycemia and glyco-

suria. Symptoms had disappeared by the time he arrived, and glucose tolerance tests were normal. It seems possible that the patient had functional hypoglycemia.

Patients With Hemolytic Disease.—It is evident from the graphic data shown in Fig. 1 that there is some correlation between reticulocytosis and erythrocyte glycolysis in patients with hemolytic disease. The glycolytic rate is approximately twice that of normal when the reticulocyte count is 10 per cent. From this it can be computed that the glycolytic rate increases by 4 mg. per 100 ml. per hour with each percentage increment in reticulocytes. If similar deductions hold true for normal cells with an average reticulocyte count of 1 per cent, the reticulocytes contribute 10 per cent to the glycolysis of normal blood. Bartlett and Marlow[6] estimated that 0.5 per cent reticulocytes in normal blood contributed only 2 per cent of the total glycolytic activity of whole blood. Selwyn and Dacie[7] estimated that the glycolytic rate of reticulocytes is approximately six times greater than that of mature red cells.

As we have pointed out, the correlation between glycolytic activity and reticulocyte count is rather poor. Table I gives the diagnoses and reticulocyte counts of the patients with hemolytic disease. Particularly striking was a patient with homozygous hemoglobin-C disease, whose red cells showed marked glycolytic activity with only slight reticulocytosis.

TABLE I. ERYTHROCYTE GLYCOLYTIC RATES OF PATIENTS WITH HEMOLYTIC DISEASE

PATIENT	DIAGNOSIS	RETICULOCYTES	GLYCOLYTIC RATE (MG./100 ML. PACKED CELLS/HR.)
Normal range		0.5-2.0	(43 ± 8.8)
B. C. Y.	Hereditary nonspherocytic hemolytic anemia	7.2	77
V. G.	Hereditary nonspherocytic hemolytic anemia	11.0	57
J. G. B.	Hereditary spherocytosis		
	(before splenectomy)	15.0	96
	(1 month after splenectomy)	1.2	51
A. F.	Hereditary spherocytosis with carcinoma of cervix	11.0	65
L. McG.	Sickle-cell anemia	16.4	64
A. A.	Sickle-cell anemia	14.7	151
A. I.	Sickle-cell C-hemoglobin disease	5.0	71
R. J.	Homozygous C-hemoglobin disease	3.4	156
	Homozygous C-hemoglobin disease (2 wk. later)	2.5	139
S. M.	Sarcoidosis with hemolytic disease (nonimmune)	5.6	63
L. C. J.	Acquired hemolytic anemia (nonimmune) with thrombocytopenia, cause unknown	5.2	71
R. B.	Paroxysmal nocturnal hemoglobinuria	4.0	59
B.	Cirrhosis of liver with acquired hemolytic anemia (immune)	13.0	71
E. R.	Carcinoma of cervix with acquired hemolytic anemia (immune)	21.0	162
I. T.	Acquired hemolytic anemia (immune) and thrombocytopenia	8.3	71
	(After splenectomy and on cortisone)	3.7	99

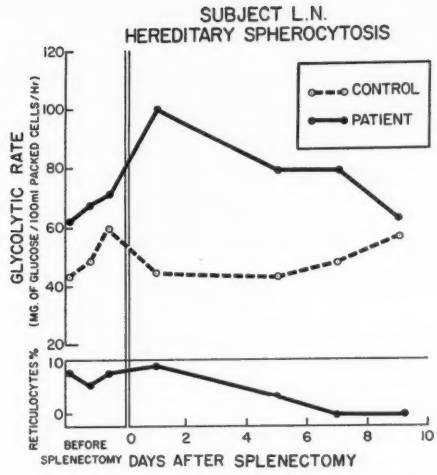

Fig. 2.—Erythrocyte glycolytic rate and reticulocyte counts following splenectomy in a patient with hereditary spherocytosis.

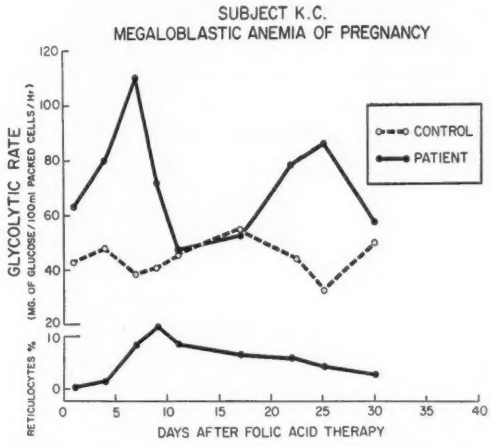

Fig. 3.—Erythrocyte glycolytic rate and reticulocyte counts during folic acid therapy (10 to 20 mg. daily) in a patient with megaloblastic anemia of pregnancy.

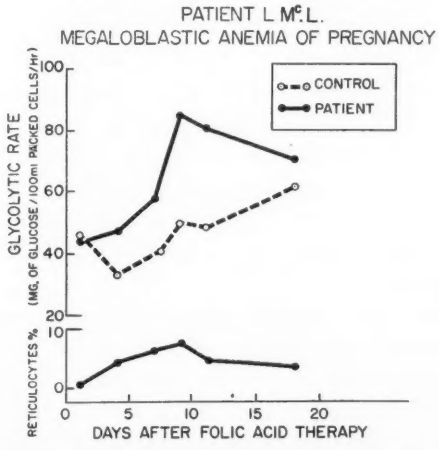

Fig. 4.—Erythrocyte glycolytic rate and reticulocyte counts during folic acid therapy (20 mg. daily) in a patient with megaloblastic anemia of pregnancy.

In an attempt to learn more about the relationship of reticulocytes to glycolysis, we performed serial determinations of glycolytic rate in three patients as reticulocyte changes occurred. Serial studies on a young soldier with hereditary spherocytosis both before and after splenectomy are shown in Fig. 2. Results of studies during folic acid therapy in two cases (sisters) of megaloblastic anemia of pregnancy are shown in Figs. 3 and 4.

DISCUSSION

Our studies of the glycolytic rate of erythrocytes from patients with hemolytic disease indicated that there is a general correlation between the glycolytic activity and the level of reticulocytes. Certain observations suggested that young red cells, even after losing their reticulum, might continue to have a relatively high metabolic activity.

The red cells of the patient with homozygous hemoglobin-C disease had marked glycolytic activity with only minimal reticulocytosis. Other studies of this patient indicated a rapid hemolytic process with an average red cell lifespan about one-eighth normal, and since his hemoglobin level fluctuated very little without transfusion, blood production must have been at least 5 or 6 times normal. This marked activity was indicated by striking red cell hyperplasia in the bone marrow, but was not accompanied by reticulocytosis. This minimal reticulocytosis in homozygous hemoglobin-C disease has been noted in reports on other patients.[8-10]

In the patient with hereditary spherocytosis, blood production presumably reverted to normal very rapidly after splenectomy, since he was not anemic and blood loss at surgery was minimal. The reticulocytes fell rapidly to normal following an immediate postoperative rise, and the curve followed the 4- to 5-day reticulocyte maturation time suggested by the studies of Young.[11] In this patient the fall of glycolytic rate to normal was not as rapid as the reticulocyte drop, and after 9 days was still outside the range of normal.

The results in the cases of megaloblastic anemia are difficult to interpret. Megaloblastic anemias are characterized by altered production of red cells accompanied by excessive hemolysis and absence of reticulocytosis. Hamilton and his co-workers,[12] on the basis of erythrocyte survival studies, have concluded that the major defect in most cases is excessive hemolysis. One patient (Fig. 3) demonstrated increased glycolysis before reticulocytosis developed, but her sister (Fig. 4) did not show this phenomenon. In Fig. 3, the later period of normal glucose utilization by the cells at a time of increased reticulocytes is not explained. Her sister demonstrated a closer correlation between glycolytic rate and reticulocytes (Fig. 4). The degree of metabolic change may vary among patients with megaloblastic anemias, depending upon the rates of red cell production and destruction.

Our results suggest that the increase in glycolytic rate seen in red blood cells taken from patients with hemolytic disease is due to young cells, both reticulated and nonreticulated. It seems possible that the glycolytic rate may give a rough index of the mean red cell age and thus, indirectly, of bone marrow activity. Reticulocytosis alone is often a poor index of erythropoietic activity,

since the number of cells showing reticulum may vary in different diseases depending upon the poorly understood mechanism of loss of reticulum by the cells, and the equally obscure mechanism of release of cells from the bone marrow.

SUMMARY

1. The mean glycolytic rate in vitro of red cells from 28 normal subjects and miscellaneous hospital patients was 43 (±8.8) mg. glucose per 100 ml. of packed cells per hour at body temperature.

2. The glycolytic rate of erythrocytes from 14 patients with hemolytic diseases was increased. In general, the increase in glycolytic rate was proportional to the number of reticulocytes present. There were some instances of increased glycolysis without marked reticulocytosis. Serial studies during subsiding reticulocytosis were performed on a patient with hereditary spherocytosis, and on two sisters with megaloblastic anemia of pregnancy. In these subjects the relationship between glycolytic rate and reticulocytes was not exact.

3. The data suggest that even in the absence of reticulocytes, the glycolytic rate in patients with hemolytic disease may be a rough index of mean red cell age and, indirectly, of erythropoiesis.

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INTRINSIC FACTOR STUDIES

III. Further Observations Utilizing the Urinary Radioactivity Test in Subjects With Achlorhydria, Pernicious Anemia, or a Total Gastrectomy

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THE diagnosis of pernicious anemia in a patient who has been treated with vitamin B_{12} or folic acid is difficult. If the patient has gastric achlorhydria, one may elect to continue vitamin B_{12} therapy or to observe the patient while withholding specific therapy. If the latter alternative is chosen, several years may elapse before the patient manifests unequivocal hematologic or neurological changes of pernicious anemia, and there is always the risk that contact with the patient will be lost, possibly resulting in serious and irreversible neurological disease. Testing the patient's gastric juice for intrinsic factor activity in a second patient with pernicious anemia in relapse is obviously not of wide applicability. At present there is no satisfactory in vitro test for intrinsic factor activity. In pernicious anemia and totally gastrectomized subjects the absorption of orally administered vitamin B_{12} is markedly less than normal. The simultaneous oral administration of intrinsic factor will enhance absorption of the vitamin, and in the past several years there have been described three in vivo techniques for the demonstration of intrinsic factor activity.[1-3] Each of these techniques utilizes radioactive vitamin B_{12} in which the Co^{60} is an integral part of the molecule, and each may be performed in patients whose pernicious anemia has been adequately treated. This is a report of data accumulated by the use of the urinary radioactivity technique[2] in a study of approximately 100 subjects.

METHODS AND MATERIALS

After the collection of a control urine specimen, 1 or 2 μg of radioactive vitamin B_{12}† ($B_{12}Co^{60}$) was administered orally in 100 ml. of water to a fasting subject. Two hours later the subject received an intramuscular injection of 1,000 μg of nonradioactive vitamin B_{12}.‡ Urine was collected quantitatively for 24 hours after the ingestion of the radioactive vitamin B_{12}. A 10 ml. aliquot of this pool of urine was assayed for radioactivity in a well-type scintillation counter, and the per cent of the orally administered radioactivity which appeared in the urine was calculated. Breakfast of toast, coffee, and fruit juice

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‡Supplied through the courtesy of Merck & Company, Inc.

was allowed 1 hour after the oral vitamin was drunk. When intrinsic factor preparations were studied they were dissolved or suspended in 100 ml. of water and the radioactive vitamin was added a few minutes before the patient drank the mixture.

Achlorhydria is here defined as the failure to find an acid reaction to Toepfer's reagent in any of 4 gastric juice specimens withdrawn at 15-minute intervals after the subcutaneous injection of 0.5 mg. of histamine phosphate.

Gastric juice to be used as intrinsic factor was collected and preserved as previously described.[4] When pooled gastric juice was lyophilized it was first dialyzed against running tap water for 24 hours. One milliliter of pooled gastric juice yielded 1.4 mg. of solids after dialyzing and lyophilizing.

Four major groups of subjects were studied:

1. Control subjects: either normal young men or hospital patients without evidence of pernicious anemia. See Table I.

2. Patients with achlorhydria but no other historical, physical, or laboratory evidence of pernicious anemia. See Table II.

3. Thirty-five patients known to have pernicious anemia (Table IV). All except 6 (Nos. 3, 13, 14, 15, 17, 21) were in hematologic remission at the time of observation. The first 22 patients in Table IV had been previously seen in this hospital at the time of a hematologic or neurological relapse. All had achlorhydria and an appropriate response to therapy. The remaining patients gave a history which left no reason for doubting the diagnosis of pernicious anemia, although they were not seen in this hospital at the time of relapse.

4. Thirteen totally gastrectomized persons. See Table V.

RESULTS AND DISCUSSION

The major portions of the data are presented in Tables I through V. Table I contains data from single observations on 18 subjects designated as controls. Data from three other control subjects are not included because they were collected before the present method of urine collection and quantitative radioactivity assay was instituted, but all three excreted gross amounts of radioactivity in the urine six to twelve hours after the oral radioactive vitamin B_{12}. Control subjects excreted from 7 to 22 per cent of the orally administered radioactivity in the urine under the conditions of these observations.

Data in Table II demonstrate that 75 per cent of the patients with achlorhydria but without other historical or laboratory evidence of pernicious anemia excreted radioactivity in the urine in amounts similar to the control subjects. Twenty-five per cent of the patients with achlorhydria excreted less radioactivity than the lowest value for a control patient. Attention is called to the observation on Patient 4 in Table II. This observation was made on a 36-year-old woman who has had gray hair and vitiligo for 20 years, but has not had a history suggestive of pernicious anemia. When the radioactive vitamin B_{12} was given with water she excreted 2.2 per cent of the radioactivity in the 24-hour urine, but in another observation, when the same dose of vitamin $B_{12}Co^{60}$ was given with an intrinsic factor preparation, she excreted 20.3 per cent of the radioactivity in the 24-hour urine. If this woman has a deficiency of intrinsic factor, and we believe she has, it may be some years before a vitamin B_{12} deficiency manifests itself clinically because the subcutaneous

TABLE I. URINARY RADIOACTIVITY IN 18 CONTROL SUBJECTS GIVEN RADIOACTIVE VITAMIN B_{12} BY MOUTH

PATIENT	AGE	SEX	DIAGNOSIS	GASTRIC ANALYSIS	$B_{12}Co^{60}$ ORAL DOSE (μG)	24-HOUR URINE VOLUME (ML.)	URINARY RADIOACTIVITY (PER CENT OF ORAL DOSE)
1. J. B.	29	M	No disease	Not done	2	790	9.0
2. E. B.	27	M	No disease	Free HCl	2	1,079	16.0
3. L. F.	32	F	Anxiety reaction	Free HCl	2	2,359	7.0
4. A. K.	65	M	Anxiety reaction	Free HCl	1	1,530	17.8
5. H. B.	70	M	Duodenal ulcer	Free HCl	1	1,800	16.6
6. S. R.	69	F	Arteriosclerosis	Free HCl	1	820	21.0
7. L. H.	75	M	Fibrosarcoma of calf	Free HCl	1	620	13.0
8. I. F.	27	F	Schizophrenia	Not done	1	1,113	11.5
9. E. M.	70	F	Basal-cell carcinoma, face	Free HCl	1	910	14.7
10. M. D.	65	F	Anxiety reaction	Free HCl	1	1,800	17.0
11. J. R.	59	M	Psychoneurosis	Free HCl	1	1,800	10.6
12. G. S.	72	M	Squamous-cell carcinoma, face	Not done	1	1,352	14.4
13. G. B.	48	F	Thyrotoxicosis	Not done	1	1,340	14.8
14. G. B.	44	M	Cholelithiasis	Not done	1	875	7.8
15. F. S.	41	F	Multiple sclerosis	Free HCl	1	3,755	15.9
16. F. V.	75	M	No diagnosis	Free HCl	1	1,600	22.2
17. G. W.	34	M	Subtotal gastrectomy	Not done	1	2,750	16.4
18. C. P.	55	M	Subtotal gastrectomy	Not done	1	1,230	10.1
						Average	**14.2**
						Range	**7-22**

TABLE II. URINARY RADIOACTIVITY IN 31 ACHLORHYDRIC SUBJECTS GIVEN RADIOACTIVE VITAMIN B_{12} BY MOUTH

PATIENT	AGE	SEX	DIAGNOSIS	$B_{12}Co^{60}$ ORAL DOSE (μG)	24-HOUR URINE VOLUME (ML.)	URINARY RADIOACTIVITY PER CENT OF ORAL DOSE
1. S. S.	44	F	No disease	1	1,135	6.9
2. P. P.	59	M	Thalassemia minor	1	875	10.6
3. J. W.	32	M	Psychoneurosis	1	2,812	18.9
4. V. R.	36	F	Vitiligo	1	3,460	2.2
5. F. C.	65	F	Neurosyphilis	1	800	9.4
6. F. F.	36	F	Irritable bowel	1	1,360	13.4
7. F. C.	65	M	Arteriosclerosis	1	512	3.2
8. L. M.	66	M	Anxiety reaction	1	2,135	9.0
9. J. W.	47	F	Anxiety reaction	1	2,569	4.2
10. E. A.	62	M	Diabetes mellitus	1	2,370	9.1
11. E. B.	29	F	Idiopathic epilepsy	1	820	7.3
12. E. R.	48	F	Psychoneurosis	1	1,375	7.0
13. M. E.	71	M	Subtotal gastrectomy	1	1,390	18.0
14. G. K.	66	M	Benign gastric ulcer	1	1,530	4.4
15. J. K.	68	F	Hypertension	1	1,760	15.1
16. H. H.	54	M	Chronic bronchitis	1	650	29.5
17. T. K.	50	F	Myeloradiculitis	1	3,880	8.7
18. U. K.	64	M	Anxiety reaction	1	1,700	20.7
19. B. Z.	34	F	Chronic alcoholism	1	1,400	13.7
20. K. G.	64	F	Chronic cholecystitis	1	1,100	21.6
21. W. C.	68	M	Gastric adenocarcinoma	1	540	27.2
22. I. B.	45	F	Anxiety reaction	1	600	17.3
23. C. S.	74	F	Basal-cell carcinoma, face	1	920	24.0
24. G. M.	53	M	Duodenal diverticulum	1	1,900	22.0
25. S. R.	39	F	Multiple sclerosis	1	1,840	4.1
26. F. D.	66	F	Leukoplakia of tongue	1	570	3.3
27. J. R.	79	M	Gastric polyps	1	750	3.5
28. E. M.	59	M	Hypernephroma	1	2,040	7.2
29. F. W.	60	M	Arteriosclerotic myelopathy	1	690	9.1
30. M. P.	74	F	Gastric adenocarcinoma	1	1,250	6.0
31. W. D.	45	M	Subtotal gastrectomy	1	1,520	3.8
					Average	11.6
					Range	2.2-29.5

administration of 1,000 μg of B_{12} on two occasions may leave her with a sufficient quantity of the vitamin for several years. One other patient (No. 16, Table II) with achlorhydria and vitiligo excreted 29.5 per cent of the radioactivity in the urine in 24 hours.

Preliminary evidence that the urinary radioactivity is due to intact vitamin $B_{12}Co^{60}$ has been presented in an earlier paper in this series.[2] MacLean and Bloch[5] have shown that the chromatographic mobility of the urinary radioactivity is identical to vitamin B_{12}.

If the urinary radioactivity test is to be considered of value in assessing a subject's ability to absorb vitamin B_{12}, the test must provide reasonably similar results when duplicate observations are made under similar conditions in the same subject. The data in Table III, representing such duplicate observations, demonstrate a reasonable degree of reproducibility. One may conclude, therefore, that a gross difference between two observations in a single patient is due to variations in the procedure. If only one step in the procedure has been changed (e.g., the addition or withholding of intrinsic factor), it is evident that this single change is causing the difference in urinary radioactivity.

TABLE III. DUPLICATE URINARY RADIOACTIVITY TESTS IN 11 SUBJECTS

PATIENT	DIAGNOSIS	URINARY RADIOACTIVITY, (PER CENT OF ORAL DOSE)	
		TEST A	TEST B
1. W. D.	Subtotal gastrectomy	3.4	3.8
2. H. E.	Achlorhydria	23.0	31.5
3. J. R.	Gastric polyps	3.3	3.5
4. M. J.	Pernicious anemia*	17.7	19.3
5. I. S.	Total gastrectomy	1.8	3.6
6. E. J.	Total gastrectomy	0	0.1
7. F. D.	Pernicious anemia	0.3	0.3
8. G. B.	Cholelithiasis	7.6	7.8
9. E. M.	Hypernephroma	7.2	10.2
10. M. P.	Total gastrectomy*	5.9	4.9
11. F. D.	Achlorhydria, leukoplakia of tongue	3.3	3.6

*These patients were given intrinsic factor with the oral vitamin $B_{12}Co^{60}$ but in each patient, except No. 7, Test B is a duplicate of Test A. Patient No. 7 received vitamin $B_{12}Co^{60}$ and boiled gastric juice in Test A, while in Test B the vitamin was given with water.

Data in Table IV show that 35 patients with pernicious anemia excreted little or no detectable radioactivity in the urine unless a source of intrinsic factor was given orally and simultaneously with the radioactive vitamin B_{12}. The maximum urinary radioactivity excreted by a pernicious anemia patient without intrinsic factor was 2.3 per cent, while the minimum for any control subject was 7 per cent. It is obvious that deficiency of intrinsic factor must exist in different degrees, and it is well known that psychologic, social, and economic factors appear to precipitate the clinical picture of pernicious anemia in some patients. It is likely that dental extractions, the death of a spouse, or the loss of one's home reduce the dietary intake of vitamin B_{12} below the point at which the patient's greatly diminished supply of intrinsic factor can effect sufficient absorption of vitamin B_{12}. It is also possible that the minimal

TABLE IV. URINARY RADIOACTIVITY IN 35 PATIENTS WITH PERNICIOUS ANEMIA GIVEN RADIOACTIVE VITAMIN B_{12} BY MOUTH

TABLE IV. URINARY RADIOACTIVITY IN 35 PATIENTS WITH PERNICIOUS ANEMIA GIVEN RADIOACTIVE VITAMIN B_{12} BY MOUTH

PATIENT AGE SEX	WITHOUT INTRINSIC FACTOR			WITH INTRINSIC FACTOR			
	B_{12} Co^{60} ORAL DOSE (μG)	24-HOUR URINE VOLUME (ML.)	URINARY RADIOACTIVITY (PER CENT OF ORAL DOSE)	B_{12} Co^{60} ORAL DOSE (μG)	24-HOUR URINE VOLUME (ML.)	URINARY RADIOACTIVITY (PER CENT OF ORAL DOSE)	TYPE OF INTRINSIC FACTOR USED
1. A. J. 66 F	1.5	2,080	0.7	2	1,404	6.0	50 ml. NNHGJ‡
2. A. M. 55 M	2	1,056	0.2*	2	1,126	9.0	100 ml. NNHGJ
3. F. D. 67 M	2	3,239	0.3	2	1,791	9.0	100 ml. NNHGJ
4. G. B. 55 M	2	2,747	0	2	2,133	13.7	100 ml. NNHGJ
5. E. H. 49 F	2	2,145	0	2	1,398	6.3	25 ml. NNHGJ
6. A. M. 65 M	2	815	0*	1	685	13.3	50 ml. NNHGJ
7. J. B. 82 M	1	2,760	0†	1	1,830	6.4	45 ml. NNHGJ
8. F. G. 64 M	1	1,350	0.5	1	1,070	8.2	NHGJ fraction
9. A. G. 65 M	1	2,330	2.3	1	2,717	9.8	500 mg. IF§
10. J. R. 60 M	1	710	0.4*	1	743	8.5	25 ml. NNHGJ
11. J. L. 68 M	1	1,070	1.1*	1	1,448	15.7	25 ml. NNHGJ
12. E. S. 60 M	1	1,734	2.0	1	750	4.6	35 mg. Ly.GJ‖
13. J. F. 46 M	1	610	0.3	1	1,325	8.2	9 mg. IF
14. T. J. 55 M	1	2,925	0	1	2,790	16.5	15 mg. IF
15. R. M. 45 M	1	1,050	0.5	1	1,850	9.0	100 ml. NNHGJ
16. M. N. 74 F	1	1,600	1.4	1	1,050	10.1	2 mg. IF
17. M. J. 60 F	1	980	0.3	1	2,200	19.3	200 ml. NNHGJ
18. J. G. 63 F	15	1,100	0.3	15	980	5.1	75 mg. IF
19. B. S. 24 M	1	760	0.1	1	1,775	7.8	141 mg. Ly.GJ
20. H. H. 50 M	1	1,540	0	1	1,470	16.3	70 mg. Ly.GJ
21. C. B. 51 M	1	950	1.0	0.5	1,810	11.0	14 mg. Ly.GJ
22. H. M. 62 F	1	1,930	0.9	1	1,775	7.1	140 mg. Ly.GJ
23. F. A. 50 F	2	224	0.2*	2	225	3.1	100 ml. NNHGJ
24. W. S. 78 M	1	779	0.8†	2	633	30.0	25 ml. NNHGJ
25. L. D. 72 F	2	1,430	0	1	1,232	12.0	50 ml. NNHGJ
26. M. N. 69 F	1	718	1.0	1	1,021	7.4	50 mg. IF
27. G. B. 41 F	1	1,820	0	1	1,395	3.4	10 mg. IF
28. E. F. 66 F	1	555	1.4	1	310	6.1	25 ml. NNHGJ
29. C. V. 68 M	1	1,588	0.3†	1	1,520	8.1	5 mg. IF
30. J. B. 51 M	1	920	1.0	1	900	8.5	NHGJ fraction
31. E. H. 60 M	1	1,475	0.8	1	500	10.4	1 mg. IF
32. E. G. 65 M	1	1,200	1.1	1	1,250	15.2	7 mg. IF
33. M. W. 73 F	1	1,050	0	1	630	6.8	35 mg. Ly.GJ
34. P. S. 28 F	1	765	0.1	1	565	8.4	14 mg. Ly.GJ
35. S. H. 74 M	1	2,445	1.4	1	2,560	9.6	35 mg. Ly.GJ
		Average	0.6		Average	10.0	
		Range	0-2.3		Range	3.1-30.0	

*Oral $B_{12}Co^{60}$ plus boiled normal human gastric juice.

†Oral $B_{12}Co^{60}$ plus inactive intrinsic factor preparation from hog intestinal mucosa.

‡NNHGJ—Neutralized normal human gastric juice or active fraction.

§IF—Intrinsic factor preparation from hog duodenal or gastric mucosa. Supplied by Armour Laboratories except that the preparation used in Patient No. 26 was furnished by Merck & Company, Inc.

‖Ly.GJ—Lyophilized NHGJ (1.4 mg. Ly.GJ = 1 ml. NHGJ).

TABLE V. URINARY RADIOACTIVITY IN 13 TOTALLY GASTRECTOMIZED PATIENTS GIVEN RADIOACTIVE VITAMIN B_{12} BY MOUTH

		WITHOUT INTRINSIC FACTOR			WITH INTRINSIC FACTOR			
PATIENT	AGE SEX	$B_{12}Co^{60}$ ORAL DOSE (μG)	24-HOUR URINE VOLUME (ML.)	URINARY RADIOACTIVITY (PER CENT OF ORAL DOSE)	$B_{12}Co^{60}$ ORAL DOSE (μG)	24-HOUR URINE VOLUME (ML.)	URINARY RADIOACTIVITY (PER CENT OF ORAL DOSE)	TYPE OF INTRINSIC FACTOR USED§
1. E. J.	53 F	1	400	0	1	1,460	12.3	10 ml. NNHGJ
2. E. K.*	66 M	1	2,410	0	1	820	7.5	10 ml. NNHGJ
3. S. K.†	62 F	1	1,226	1.0	1	570	6.3	25 ml. NNHGJ
4. M. P.*	74 F	1	630	0.1	1	900	5.9	10 ml. NNHGJ
5. I. S.*	57 F	1	750	0.1	1	950	3.6	10 ml. NNHGJ
6. E. F.†	50 M	1	1,175	0.6	1	1,302	9.1	NHGJ fraction
7. N. C.	65 M	1	900	0.3	1	1,600	18.2	14 mg. Ly.GJ
8. S. B.	56 M	1	1,155	0.3	1	690	6.4	7.5 mg. IF
9. P. S.	64 M	1	415	0.3	1	350	8.8	100 ml. NNHGJ
10. L. H.*	60 F	1	435	0	1	650	3.3	35 mg. Ly.GJ
11. G. M.†	59 M	1	675	0				
12. A. B.	72 F	1	870	0‡	1	935	5.3	10 ml. NNHGJ
13. P. J.*	63 M	1	2,530	0.2‡	1	3,135	4.8	NHGJ fraction
			Average	0.2		Average	7.6	
			Range	0-1.0		Range	3.3-18.2	

*Patients with achlorhydria preoperatively.
†Patients with free gastric acid preoperatively.
‡Oral $B_{12}Co^{60}$ plus inactive intrinsic factor preparation.
§Intrinsic factor types: See footnote, Table IV.

gastric secretion of intrinsic factor in such patients is further diminished by certain forms of emotional stress. It is, therefore, quite reasonable to assume that one will observe achlorhydric patients who can absorb very little vitamin B_{12} but who have not yet exhibited signs and symptoms of pernicious anemia. The relatively long biologic half life of vitamin B_{12}[6, 7] suggests that the development of clinical signs of pernicious anemia may require many months of subminimal vitamin B_{12} absorption.

The amount of radioactivity appearing in the urine of pernicious anemia patients given intrinsic factor is in some instances below values observed in the control subjects. This may be the result of a suboptimal quantity of intrinsic factor being given to the patient, or some pernicious anemia patients may have a diminished intestinal permeability.

Occasionally patients who have had their entire stomach removed develop megaloblastic anemia. Patient 13 in Table V was observed during two episodes of macrocytic megaloblastic anemia 6½ and 7 years after his total gastrectomy. In each instance vitamin B_{12} injections brought a hematologic response. In recent months Halsted and co-workers[8] have reported data on 11 totally gastrectomized patients studied by the fecal radioactivity technique. Their results and the data in Table V indicate that such patients are able to absorb very little of the administered vitamin B_{12}. These patients are quantitatively similar to pernicious anemia patients in their inability to absorb vitamin B_{12} from the intestine. The addition of intrinsic factor causes an increased absorption. The stomach apparently is the only source of intrinsic factor in the human alimentary tract. Patients 5 and 8 in Table V had an end-to-end esophagoduodenostomy, but the other 11 patients had an esophagojejunostomy with a blind duodenal loop. The two gastrectomized subjects having no blind loop of duodenum were as ineffective in absorbing vitamin B_{12} as were those with the blind loop. Therefore, one may assume that gastrectomized patients with an esophagojejunostomy are not analogous to those patients described in the literature under the term "intestinal blind loop megaloblastic anemia."[9-11] Patients 2 and 4 were studied 9 days postoperatively, and each was unable to absorb vitamin B_{12} at this time. This result would be anticipated from previous work of Castle and Ham.[12] One patient excreted 6 per cent of the radioactivity in the urine before gastrectomy (Table II, No. 30) and 0.1 per cent after (Table V, No. 4).

SUMMARY AND CONCLUSIONS

When *radioactive vitamin B_{12}* is administered by mouth to subjects who can absorb it, radioactivity will appear in the urine if the subject is "flooded" with an intramuscular injection of *nonradioactive vitamin B_{12}*. Using this urinary radioactivity technique, 97 subjects were studied for their ability to absorb vitamin B_{12}.

Eighteen control subjects excreted from 7 to 22 per cent of the orally administered radioactivity in the urine in 24 hours, with an average of 14.2 per cent.

Thirty-one achlorhydric subjects with no previous history suggestive of pernicious anemia excreted from 2.2 to 29 per cent of the radioactivity, with an average excretion of 11.6 per cent.

Thirty-five patients known to have pernicious anemia excreted 0 to 2.3 per cent of the radioactivity in the urine when the radioactive vitamin B_{12} was given without added intrinsic factor. When the same dose of the radioactive vitamin was given with an intrinsic factor preparation these patients excreted from 3.1 to 30 per cent of the radioactivity in the urine, with an average of 9.8 per cent.

Thirteen totally gastrectomized subjects were entirely similar to the pernicious anemia patients as regards their inability to absorb vitamin B_{12}.

The urinary radioactivity test is useful in the diagnosis of pernicious anemia. The test is of aid in estimating intrinsic factor activity in preparations of unknown activity.

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CHARACTERISTICS OF THE MICROCOCCAL NASAL CARRIER STATE AMONG HOSPITAL PERSONNEL

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SEVERAL studies,[1, 2] particularly those of Miles and co-workers,[2] have indicated that certain individuals are more likely than others to harbor coagulase-positive *Micrococcus pyogenes* var. *aureus* for prolonged periods of time.

During the course of epidemiologic studies of micrococcal infections in a hospital community, we had the opportunity to study some of the characteristics of the carrier state among ward personnel. In this paper we are reporting data which add to the material previously reported by others on the occurrence and duration of the carrier state and on the relationship of these to individual host differences. It has been confirmed that certain personnel carry potentially pathogenic organisms, as evidenced by the production of coagulase, for prolonged periods and therefore constitute a potential hazard to the patients. Moreover, among the personnel who are persistent carriers there are two groups: one in which the same strain is consistently present and the other in which many changes of strain occur.

METHOD

All studies were performed on the personnel of the Municipal Contagious Disease Hospital of Chicago during their tenure on the wards. Nasal and pharyngeal cultures were obtained from each person at monthly intervals wherever possible. However, because of vacations, illnesses, and similar interfering factors, only 996 nose and throat cultures were obtained in 1,634 personnel months or an average of 1 culture per 1.7 months of observation. All cultures were obtained by thoroughly swabbing the appropriate area with a sterile cotton swab and immediately streaking the swab on a blood agar plate. Several colonies of each micrococcus species were subcultured for further study. After a "pure" culture was obtained coagulase tests were done. The production of alpha hemolysin by some strains was also determined. Classification of all coagulase-positive organisms was attempted by phage lysis. During the first eight months of the study the minimal concentration of three or more antibiotics which would inhibit the growth of each isolate was determined by serial twofold dilutions. The three antibiotics most commonly employed were chlortetracycline, penicillin, and streptomycin. During the last eighteen months the latter was replaced by erythromycin. On many organisms tests were done with other antibiotics as well.

In recording the results, organisms were considered to be the same if they were of the same phage group without major discrepancies in their pattern

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of lysis. If they were not typable they were considered the same if the coagulase tests were consistent and the minimal inhibiting concentration of penicillin varied no more than 3 tubes and the other antibiotics varied no more than two tubes. The limits have been shown to be 80 per cent reliable in confirming identity and 68 per cent reliable in establishing a difference among strains isolated serially in the same person.[4] These criteria, then, are more likely to identify different strains as being the same than vice versa.

RESULTS

As can be seen in Table I, there were 217 persons in the series. Among these, 161 had more than one culture performed, 4 of whom have been observed for over twenty-four months, 17 others for over eighteen months, and 45 in addition for more than one year. In approximately one-half (51.1 per cent) of the examinations a strain of micrococcus was found and one-third (34 per cent) of these were coagulase-positive.

TABLE I. INCIDENCE OF POSITIVE NOSE AND/OR THROAT CULTURES FOR MICROCOCCI AMONG HOSPITAL PERSONNEL OBSERVED REPEATEDLY

DURATION OF OBSERVATION IN MONTHS	NUMBER OF EMPLOYEES CULTURED	NUMBER OF CULTURES	POSITIVE CULTURES			
			NUMBER	PER CENT	COAGULASE-POSITIVE	COAGULASE-NEGATIVE
Over 24	4	53	22	41.5	10	12
18-23.3	17	186	105	56.0	41	64
12-17.9	45	409	200	48.9	62	138
6-11.9	39	196	89	45.4	27	62
Under 6	56	152	89	58.6	30	59
Only one culture	56	56	33	59.0	14	19
Total	217	1,052	538	51.1	184	354

Fig. 1 shows that with the passage of time, the percentage of individuals who had not been shown to harbor a strain of micrococcus on at least one examination steadily decreased, so that no one who had cultures taken over more than 18 months remained free of micrococci. On the other hand, even among the personnel with the longest period of observation, coagulase producers were never observed at any time in 23.8 per cent. Both coagulase producers and nonproducers were isolated at some time from all of the remainder. Only in the group which was observed for less than six months was there an appreciable number who harbored only coagulase-producing micrococci.

That the carrier state of the same strain of micrococcus is quite transient is seen in Fig. 2. For example, only 37.8 per cent of personnel who originally had coagulase-positive organisms still had the same strain at the end of one month. Similarly, only 31.7 per cent of the negative strains persisted for one month. From that time on the percentage loss of a given strain was directly proportional to the passage of time. The slopes of the lines indicated 1.3 per cent and 1.1 per cent rates of loss per month among carriers of coagulase-positive and coagulase-negative micrococci, respectively. From the high coefficient

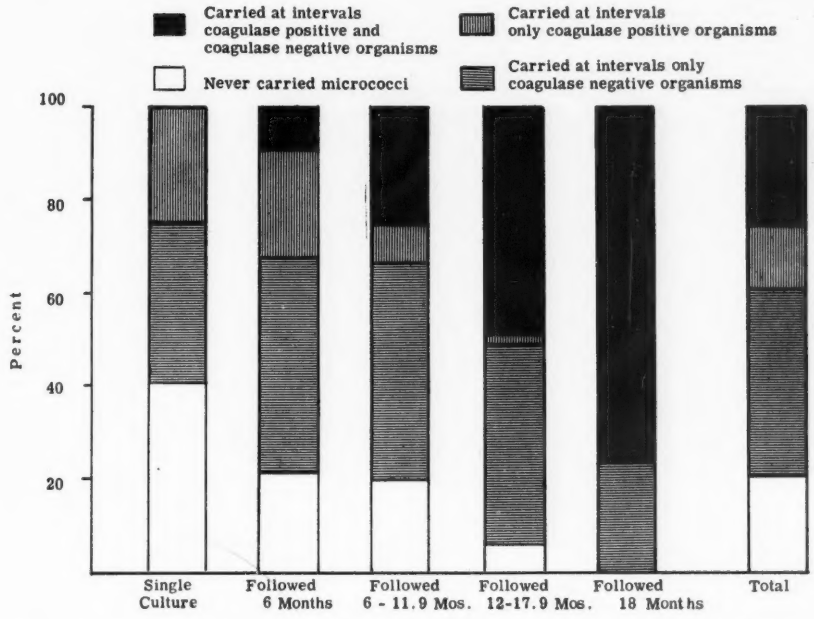

Fig. 1.—Per cent of personnel who carried micrococci in noses at some time during the study.

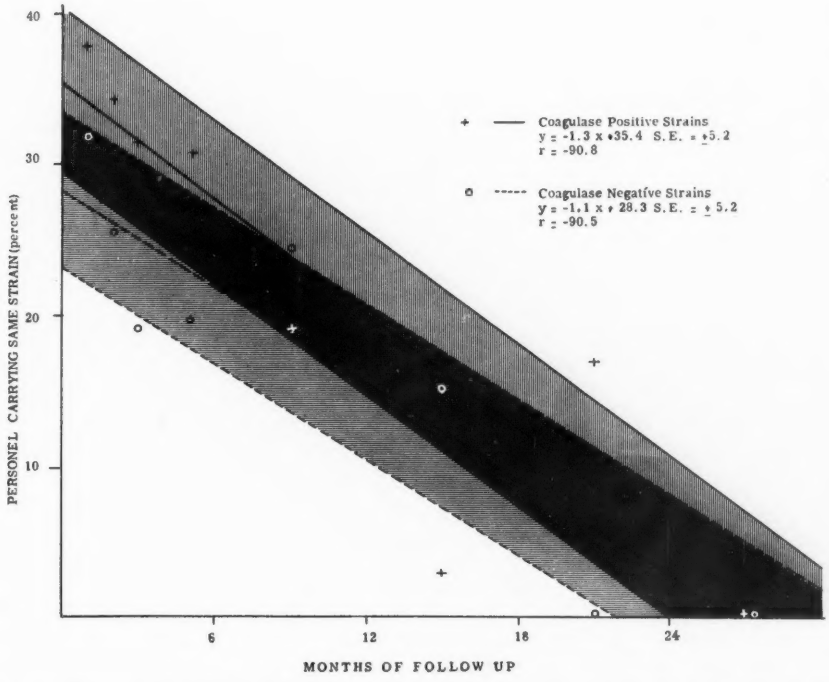

Fig. 2.—Persistence of specific strains of micrococci in nose of persons cultured at frequent intervals.

TABLE II. INCIDENCE OF POSITIVE CULTURES AND NEW STRAINS OF MICROCOCCI AMONG PERSONNEL HAVING MORE THAN ONE CULTURE

	NO. OF PERSONS	TOTAL FOLLOW-UP CULTURES	POSITIVE FOLLOW-UP CULTURES															
			ALL STRAINS								ONLY NEW STRAINS							
			TOTAL		COAGULASE-POSITIVE			COAGULASE-NEGATIVE			TOTAL		COAGULASE-POSITIVE			COAGULASE-NEGATIVE		
						PER CENT			PER CENT					PER CENT			PER CENT	
			NO.	PER CENT	NO.	POSITIVE CULTURES	TOTAL	NO.	POSITIVE CULTURES	TOTAL	NO.	PER CENT	NO.	POSITIVE CULTURES	TOTAL	NO.	POSITIVE CULTURES	TOTAL
Personnel in whom initial culture contained coagulase-positive micrococci	35	254	155	61	75	48	29.5	80	52	31.5	116	45.7	36	31	14.1	80	69	31.6
Personnel in whom initial culture contained coagulase-negative micrococci	59	252	124	49	14	11	5.5	110	89	43.5	85	33.8	14	16.5	5.6	71	83.5	28.2
Personnel in whom initial culture was negative	57	386	131	34	36	27	9.5	95	73	24.5	100	25.9	24	24.0	6.2	76	76.0	19.7

of correlation it is evident that after the first month the linearity of the function is a good approximation of the rate of disappearance of these organisms. It is also apparent that there is essentially no difference between coagulase-negative and coagulase-positive organisms in this respect.

In Table II it is seen that among the personnel whose initial cultures revealed coagulase-positive organisms (coagulase-positive group) follow-up cultures were positive more frequently (61 per cent) than were cultures on those who originally carried coagulase-negative (49 per cent) micrococci (coagulase-negative group) or those in whom the first culture was negative (34 per cent) (noncarrier group). The difference between the first and second, first and third, and second and third groups was significant with chi square values of 6.7, 44.2, and 15.7, respectively. Similarly the coagulase-positive group had a much higher incidence of coagulase-positive organisms subsequently isolated, whether calculated on the basis of total cultures (29.5 per cent) or of positive follow-up cultures (48 per cent), than did the coagulase-negative group, 5.5 per cent and 11 per cent, respectively. The differences were significant, with chi square values of 49.0 and 41.3. The values for the coagulase-positive group were also greater than for the noncarrier group, with chi square of 44.5 and 13.2 for the differences. In addition, the incidence of coagulase-positive organisms in the positive follow-up cultures of the coagulase-negative group was significantly less than for the noncarrier group (chi square 10.4). On the basis of total cultures, however, the difference was not significant.

In addition, if only new strains are considered, the coagulase-positive group had a higher incidence of new strains implanting (45.7 per cent) than either the coagulase-negative group (33.8 per cent) or the noncarrier group (25.9 per cent). The differences between the coagulase-positive and the other two groups were significant (chi square equals 22.3 and 7.4, respectively), as was the difference between the coagulase-negative and noncarrier group (chi square 4.57). On the basis of total cultures, more new coagulase-positive strains were found in the coagulase-positive group (14.1 per cent) than in the coagulase-negative group (5.6 per cent) or noncarrier group (6.2 per cent). These differences were significant, with chi square values of 11.2 and 10.6, respectively. However, on the basis of total new strains implanted, significantly more new coagulase-positive strains implanted in the coagulase-positive group than in the coagulase-negative group, with chi square 5.8. On the other hand, more new coagulase-negative strains implanted in both the coagulase-positive and coagulase-negative groups than in the noncarrier group. The chi square values for these significant differences were 11.5 and 6.2.

DISCUSSION

It has been shown that micrococcal carriers are frequent, and in the case of aureus variety, the nasal cavity is the most frequent site of infection. The skin, particularly that of the hands and face, is seemingly secondarily involved.[1, 2] Gillespie and co-workers[1] first indicated that some persons are more likely than others to carry staphylococcus persistently. Miles and his group[2]

expanded these findings and showed that among persons followed for 14 months, 10 per cent were persistently negative and 15 to 20 per cent persistently positive for coagulase-producing staphylococcus. However, they made no mention of strain identification.

In addition, it has previously been recognized that carriers among hospital personnel caring for patients are most important in the transmission of antibiotic-resistant micrococci.[4-13]

The control of the carriers of antibiotic-resistant micrococci has therefore become a problem of importance and one to which the data obtained in this study contribute some information. It is apparent from the data reported that at any one time approximately 25 per cent of the personnel carried coagulase-positive potential pathogens and another 40 per cent carried other micrococci. Sixty to 70 per cent of these persons were transient carriers, in the sense that the original organism was permanently lost within one month. The remainder, however, were more persistent carriers in whom the original strain was lost at a rate of about 1 to 1.5 per cent per month. Prolonged carriers such as these make the eradication of foci of antibiotic-resistant strains most difficult, and similar carriers undoubtedly disseminate resistant strains in the community.[14]

On the other hand, it is also evident that at least 75 per cent of the personnel, if observed over a period of months, will ultimately be shown to be at least transient carriers of coagulase-positive micrococci and all may carry the less pathogenic forms. This cumulated percentage of carriers is believed to represent primarily new implantations rather than failure to demonstrate a carrier state on the single culture. Cultures at more frequent intervals up to three a week have been made on patients, family contacts, and personnel, during the first four years. In all these situations the shorter the interval between cultures, the more likely the cultures were to be repeatedly negative or repeatedly positive and, among these latter, repeatedly positive for the same phage type. Moreover, duplicate cultures taken early in the course of these studies indicated that negative and positive cultures were duplicatable over 90 per cent of the time.

Even though the cumulative figures indicate that from a qualitative point of view the majority of personnel will at some time carry the organisms, nevertheless, the data in our study indicate that when a person is found to harbor coagulase-positive organisms on a given examination, during the next year to eighteen months he is much more likely to continue to carry such organisms than persons simultaneously found to harbor coagulase-negative organisms or no micrococci. We have demonstrated clearly that this is not due solely to the persistence of the original strain in the chronic carriers but also to the implantation of new strains of coagulase-positive staphylococcus. Thus, quantitatively, there is a definite difference among personnel as to their carrier state. This predilection of certain persons to carry and/or have implanted in their upper respiratory tracts the more pathogenic organisms over a period of time exceeding one year indicates that they are most important in the spread

of antibiotic-resistant pathogens. The evidence that other personnel tend to carry selectively coagulase-negative micrococci was not as conclusive, but new strains of these organisms were implanted to a greater degree in persons found to harbor a micrococcus than in those whose cultures were negative.

The reason for the increased susceptibility of certain persons to infection by micrococci is not apparent from this study. An analysis of environmental factors showed that the distribution of the "susceptible" personnel was not related to ward assignment, place of residence, or age. Some host factor may be important in producing this susceptibility. The fact that certain individuals do selectively carry the pathogenic strains may also be pertinent to the problem of repeated furuncles and similar infections in some patients.

SUMMARY

1. One hundred sixty-one persons working on the wards of a hospital have had repeated nose and throat cultures taken and examined for micrococci repeatedly at monthly intervals. One hundred five persons have been observed over six months and 66 over one year.

2. Initially, 59 per cent harbored micrococcus of all kinds and 25 per cent coagulase-positive strains. Of the group on whom repeated cultures were made, 100 per cent carried micrococci on one or more occasions and 76 per cent had at least one coagulase-positive strain.

3. Two-thirds of the personnel who carried micrococci lost the specific strain within one month but the remaining one-third lost the original strain slowly, at the rate of 1-1.5 per cent per month.

4. Persons who originally carried coagulase-positive strains continued to carry these strains or implanted new coagulase-positive strains more frequently than persons who originally had coagulase-negative strains or negative cultures. The persons who originally had coagulase-negative strains maintained these and tended to implant new coagulase-negative strains more often than the group with negative cultures.

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EXPERIMENTAL STUDIES ON BACTERIAL CONTAMINATION OF BANK BLOOD

II. Observations on Bacteremia in Mice and the Influence of Antibiotics in Preventing Death

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IN A previous paper[1] it was shown that the shocklike state and death of mice which followed the injection of large numbers of living coliform bacteria appeared to be closely related to the number of viable organisms injected. It was also demonstrated that antibiotics would protect the majority of animals from a lethal dose of living bacteria provided the drugs were given within a short time after the injection of the organisms. It is the purpose of the present communication to present data on the extent of bacteremia which occurs in mice following the injection of large numbers of ordinarily nonpathogenic living gram-negative bacteria and the influence and relationship of antibiotic therapy to bacteremia and the time of death.

METHODS AND MATERIALS

Bacteria.—The organism used throughout this study, *Escherichia intermedium*, strain No. 4, was the same as used in the previous investigation.[1] It was sensitive* in vitro to 2.5 μg per milliter of streptomycin and 10 μg per milliter of chloramphenicol or oxytetracycline. For inoculation into mice, 24-hour broth cultures were centrifuged, the supernate discarded and the sedimented cells resuspended in 1/10 the original volume in saline solution. All suspensions were injected intraperitoneally in a volume of 0.5 ml.

Mice.—White Swiss mice of both sexes and of heterozygous stock, weighing 20 grams, were employed.

Effects of Injection of Bacteria Into Mice.—After intraperitoneal injection of 10^9 living coliform bacteria, there was a latent period of approximately 1 hour during which the animal appeared normal. During the next 2 to 4 hours lethargy, ruffled fur, labored respiration, and increasing signs of generalized weakness occurred. Moribund animals were ataxic and frequently had diarrhea or blood-tinged urine. Death usually occurred within 5 or 6 hours after the injection.

Antibiotics.—The antibiotics used in this study were commercial dihydrostreptomycin sulfate (Merck), oxytetracycline hydrochloride† (Pfizer), and synthetic chloramphenicol‡ (Parke, Davis). All antibiotic solutions were prepared with sterile saline solution for each experiment. Dilutions of the stock solutions to the desired final concentration were made immediately before use. All antibiotics were injected subcutaneously in a volume of 0.2 to 0.5 ml.

Bacteremia Studies.—The extent of bacteremia in mice at various intervals following the injection of living coliform organisms was estimated as follows. At each interval, 2

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*Minimum concentration of the antibiotic in broth causing complete inhibition of growth and death of the organisms within 48 hours.
†Supplied by Dr. F. F. Fink.
‡Supplied by Dr. G. Rieveschel.

mice were anesthetized with ether and secured by the limbs to a metal dissecting tray. The chest was thoroughly swabbed with iodine and the heart exposed. Blood was withdrawn from the heart with a 20 gauge needle attached to a tuberculin syringe previously moistened with 0.3 per cent citrate solution. One-tenth milliter of heart blood was added to 0.9 ml. of 0.3 per cent citrate solution in order to prevent coagulation. Further tenfold dilutions of blood were made in sterile water, and aliquots of the appropriate dilutions were plated in duplicate on Proteose Peptone No. 3 agar (Difco). The plates were incubated at 37° C. for 48 hours and the colonies counted. The number of viable organisms present in the blood, as estimated by the plate count, of any pair of animals sacrificed at the same time was usually in fairly close agreement, that is, within a tenfold range. Occasionally, however, there was a 2 or 3 log difference in the extent of bacteremia between identically treated animals. The numbers of organisms recorded as being present in the blood at any given time are, therefore, not intended to be exact, but are an average of the findings of 2 or more experiments.

RESULTS

Previous studies[1] have shown that the toxicity of contaminated blood or saline suspensions of coliform bacteria for mice was closely related to a critical number of organisms injected. Thus, 10^9 viable cells caused prostration and death of all animals within 6 to 18 hours, while 10^8 organisms produced only transitory signs of illness, and most animals survived. In view of the wide disparity between the clinical effects caused by a tenfold difference in the size of the inoculum, it seemed worth while to investigate the extent of bacteremia following the injection of different numbers of bacteria. Accordingly, the cells from a 24-hour broth culture of *Esch. intermedium* were centrifuged and resuspended in one-tenth the original volume in saline solution. The undiluted suspension (containing $10^{9.3}$ viable cells per milliliter) and the suspension diluted 1:10 and 1:100 was then injected intraperitoneally into 3 groups of 35 mice each. Fifteen animals from each group were kept under observation to determine the death rate. From the remaining mice in each group 2 animals were removed at frequent intervals, anesthetized with ether, and bled from the heart. Dilutions of the blood were plated in duplicate on nutrient agar. Because of technical difficulties, no attempt was made to estimate bacteremias of less than 100 organisms per milliliter of blood, and we have arbitrarily considered blood containing less than this number of bacteria to be sterile.

The results of a typical experiment are shown in Fig. 1. The injection of 10^9 viable organisms rapidly produced a massive bacteremia of more than 10^7 bacteria per milliliter of blood, which remained constant until the animals died 5 or 6 hours later. Bacteremias of this magnitude apparently overwhelmed the host's defenses and only relatively few of the injected bacteria were removed from the blood stream by phagocytosis and filtration. On the other hand, when 10^8 living organisms were injected, a bacteremia of approximately $10^{5.6}$ bacteria per milliliter of blood occurred 1 hour after the injection, and thereafter rapidly declined until the blood was sterile by the twelfth hour. Although all animals in this latter group appeared somewhat ill during the 12 hours after the injection, only 2 of the mice died. The injection of 10^7 bac-

teria produced a barely detectable bacteremia for only a short time. Furthermore, the animals in this last group showed no signs of illness and there were no deaths.

These data suggest that, in addition to the "toxicity" inherent in the presence of large numbers of bacteria in the tissues, the ability of the normal blood-clearing mechanisms, i.e., the reticuloendothelial system, to remove and destroy the bacteria as they gain entrance to the circulation might also play an important role in determining whether the animal lives or dies. If this is so, the administration of antibiotics might increase chances of survival from an injection of overwhelming numbers of living bacteria by inhibiting bacterial multiplication and favoring bacterial removal. To test this hypothesis the following experiments were carried out.

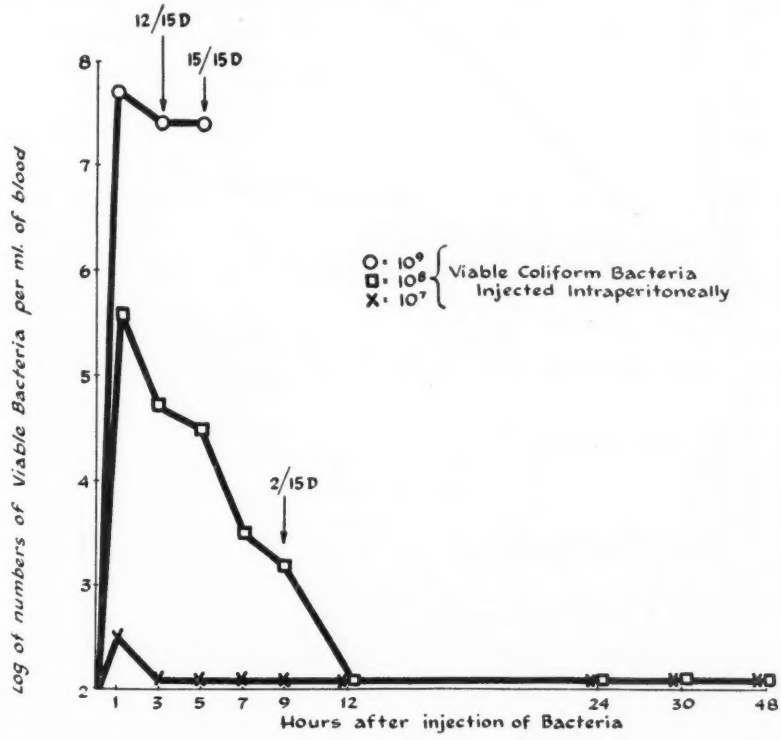

Fig. 1.—Bacteremia following injection of lethal and sublethal numbers of viable coliform bacteria in mice. Arrows indicate time of death and cumulative mortality.

The Effects of Dihydrostreptomycin on Bacteremia.—Three groups of 35 animals each were injected intraperitoneally with 0.5 ml. of a suspension of bacteria containing $10^{9.3}$ viable cells per milliliter. A single dose of 3.0 mg. of dihydrostreptomycin was administered subcutaneously to 2 groups of animals 1 and 3 hours later, respectively. A control group was injected with an equal volume of saline solution. Each group of animals was then subdivided as in previous experiments for studies on bacteremia and determination of time of death.

The results of 3 such experiments are shown in Fig. 2. Bacteremia in the untreated control group reached a peak about 1 hour after the injection of the

bacteria, as also shown in Fig. 1, and remained at the same level until all animals were dead 6 hours later. In the group given antibiotics 1 hour after the bacteria, there was an immediate and rapid decline in the number of viable organisms circulating in the blood, and by the eighth hour the bacteremia was barely detectable. However, between the tenth and tweny-fourth hours, organisms again appeared in the circulation in considerable numbers and continued to be present for some time, until the blood finally became sterile by

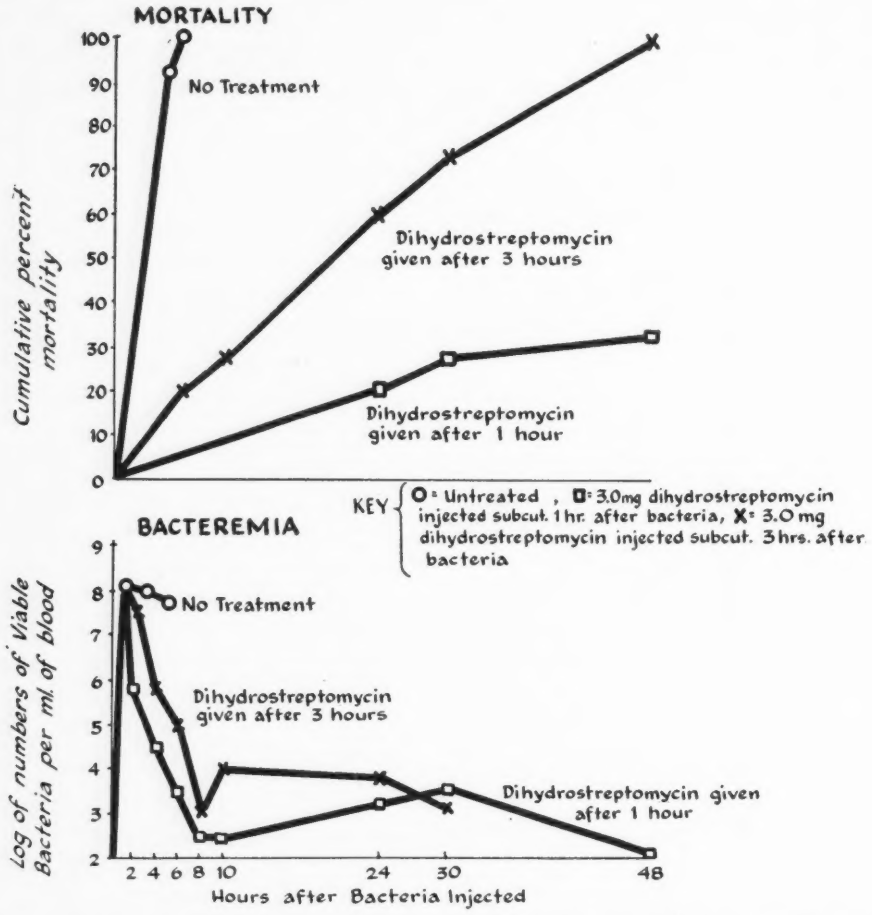
MORTALITY
Cumulative percent mortality
No Treatment
Dihydrostreptomycin given after 3 hours
Dihydrostreptomycin given after 1 hour
KEY
O = Untreated , □ = 3.0mg dihydrostreptomycin injected subcut. 1 hr. after bacteria, X = 3.0 mg dihydrostreptomycin injected subcut. 3 hrs. after bacteria
BACTEREMIA
Log of numbers of Viable Bacteria per ml of blood
No Treatment
Dihydrostreptomycin given after 3 hours
Dihydrostreptomycin given after 1 hour
Hours after Bacteria Injected

Fig. 2.—Influence of dihydrostreptomycin on bacteremia and mortality. All animals injected intraperitoneally with 10^9 viable coliform bacteria.

the forty-eighth hour. The reappearance of relatively large numbers of bacteria in the blood between the tenth and twenty-fourth hours may have been due to organisms which had survived initial contact with the antibiotic and then gained access to the blood stream when the concentration of the drug fell below effective levels. The animals in this group exhibited the same clinical picture of shock and prostration as did the animals in the untreated control group. They appeared most seriously ill between the eighth and tenth hours

at a time when only a relatively few bacteria were present in the blood. Paradoxically, by the twenty-fourth hour the clinical picture suggested that most of the animals would recover, even though bacteria circulated in the blood in considerable numbers. In the group given antibiotic therapy after a 3-hour delay, the same initial rapid fall in bacteremia occurred, but it did not quite reach as low a level as in the group treated after 1 hour. In addition, relatively large numbers of bacteria reappeared in the blood more promptly and remained at a higher level longer than in the previous group.

Comparison of the numbers of bacteria present in the blood at the time of death between the 3 groups shows that all untreated animals were dead within 6 hours, with bacteremias of approximately $10^{7.7}$ organisms per milliliter. In both groups of animals given dihydrostreptomycin most of the deaths occurred with bacteremias ranging from $10^{2.5}$ to $10^{4.0}$ organisms per milliliter. However, only 5 of 15 mice succumbed when the antibiotic was administered 1 hour after the bacteria, while all 15 animals died when therapy was delayed for 3 hours. Even though dihydrostreptomycin was unable to afford complete protection from the bacterial injection, death in most cases was delayed considerably beyond that in the untreated group.

These data indicate that the administration of dihydrostreptomycin 1 hour after the injection of a lethal dose of living coliform organisms rapidly reduced the number of viable organisms in the blood stream and prevented death in the majority of cases. However, when therapy was delayed for 3 hours, all animals eventually died, even though the bacteremia was reduced to comparatively low levels. The question then arose whether the continued presence of bacteria in the blood in this latter group, perhaps due to inadequate therapy, was responsible for the death of the animals or whether the 3-hour delay permitted sufficient time for injury to the host tissues to occur so that even adequate therapy would not have saved the animals. In order to determine which of these alternatives might be correct, experiments were carried out to learn whether intensive antibiotic therapy begun 3 hours after the bacteria were injected would completely suppress bacteremia and thereby protect the animals from death.

Effects of Prolonged Single and Combined Antibiotic Therapy on Bacteremia and Mortality.—Groups of mice were inoculated with 10^9 living coliform bacteria as previously described. Dihydrostreptomycin or dihydrostreptomycin together with chloramphenicol or oxytetracycline was administered to different groups of animals beginning 3 hours after the bacteria were injected, and continued until all animals in the group were dead or until the experiment terminated 72 hours later. Chloramphenicol and oxytetracycline were chosen for combined therapy with dihydrostreptomycin on the basis of rapid bactericidal action of the combinations in vitro. Each group of animals treated with the various antibiotics was subdivided for studies of bacteremia and time of death as previously described.

Results of these experiments are shown in Table I. Data on the extent of bacteremia have been omitted from the table because the results were essentially similar, regardless of the type of antibiotic regimen employed. Both prolonged single and combined therapy reduced the number of bacteria circulating in the blood to below detectable levels (approximately 10^2 organisms

TABLE I. INFLUENCE OF SINGLE AND COMBINED ANTIBIOTIC THERAPY ON CUMULATIVE MORTALITY OF MICE INJECTED INTRAPERITONEALLY WITH 10^9 VIABLE COLIFORM BACTERIA

HOURS AFTER BACTERIA INJECTED	CONTROLS UNTREATED (D/T*)	AMOUNT OF ANTIBIOTICS ADMINISTERED (MG. PER INJECTION†) DIHYDROSTREPTOMYCIN ONLY	D/T	DIHYDROSTREPTOMYCIN + CHLORAMPHENICOL		D/T	DIHYDROSTREPTOMYCIN + OXYTETRACYCLINE		D/T
3	0/20	3	0/20	3	1	0/20	3	1	0/20
5	10/20	2	3/20	2	1	2/20	2	1	4/20
7	18/20	2	4/20	2	1	3/20	2	1	10/20
24	19/20	3	11/20	3	1	13/20	3	1	16/20
30	—	2	12/20	2	1	14/20	2	1	--
48	—	2	14/20	2	1	15/20	2	1	18/20
72	19/20	–	16/20	–	–	18/20	–	–	20/20

*D/T = Number of animals dead/total number of animals injected, cumulative mortality.

†All antibiotics injected subcutaneously in a volume of 0.2 to 0.5 ml. Controls consisted of 3 groups of 5 mice each injected with the same drugs and doses as in the experimental group; there were no deaths or ill effects observed among the antibiotic controls.

per milliliter) in 8 hours, and thereafter either prevented their return completely or permitted only minimal numbers to appear. All bloods were sterile by 48 hours in the surviving animals. Nevertheless, the majority of mice in all groups succumbed. Thus, it appears that the shocklike state and death of mice injected with overwhelming numbers of living coliform bacteria bear little relationship to the extent of bacteremia at the time of death.

DISCUSSION

The profound physiologic disturbances and death of animals injected with dead gram-negative bacteria are attributed to the endotoxins of the organisms.[2] It has previously been shown that death due to endotoxin intoxication occurred only when a critical number of bacteria were injected.[1] In the case of coliform bacteria injected intraperitoneally into mice, a minimum of $10^{8.5}$ living cells or approximately $10^{9.5}$ dead cells were necessary to cause death. It was postulated that the critical number of living cells caused death only after sufficient multiplication had taken place in vivo to constitute a lethal dose of endotoxin, i.e., the lysis of a critical number of bacteria released a minimum lethal dose of toxic material.

In the experiments reported here there appeared to be a relationship between the efficiency of removal of bacteria from the blood stream, presumably by the reticuloendothelial system, and survival or death of the animal. For example, the intraperitoneal injection of 10^9 living coliform bacteria rapidly produced bacteremias of $10^{7.7}$ organisms per milliliter of blood, which remained constant until all animals died 5 or 6 hours later. On the other hand, 10^8 bacteria caused peak bacteremias of $10^{5.6}$ organisms per milliliter 1 hour after injection, but thereafter the number of organisms circulating in the blood rapidly declined, and by the twelfth hour the blood was sterile. The great majority of these animals survived. The apparent relationship between the inability of the reticuloendothelial system to cope with the larger number of bacteria and death of the animals may be misleading, since it was clearly shown that intensive single or combined antibiotic therapy begun 3 hours after

the bacteria were injected promptly reduced and suppressed the bacteremia, but the majority of mice died anyway. Antibiotics were much more effective in preventing death if they were administered within 1 hour after the bacteria.

Under ordinary circumstances, the reticuloendothelial system is a stable and efficient mechanism for filtering out and destroying bacteria circulating in the blood, even in the face of overwhelming infections.[3, 4] However, if it is assumed that death following the injection of huge numbers, i.e., a critical dose, of living coliform organisms is due to the *rapid* accumulation of a lethal amount of endotoxin within susceptible tissues, then the apparent failure of the reticuloendothelial system to destroy the circulating bacteria and prevent death may only reflect the fact that sufficient bacterial multiplication had occurred to build up a lethal dose of endotoxin *before* the organisms could be destroyed and endotoxin decomposed or excreted. This assumption is supported by observations that antibiotics administered 1 hour, but not 3 hours, after the bacteria were injected prevented death of the majority of animals. Thus, prompt antibiotic therapy, by suppressing bacterial multiplication, would favor the rapid destruction of the organisms and prevent the endotoxin from reaching a lethal concentration. Nevertheless, there can be little doubt that the reticuloendothelial system plays an important part in preventing endotoxin intoxication. For example, Beeson[5] and others[6] have shown that prior "blockade" of the reticuloendothelial system with Thorotrast or colloidal iron greatly enhanced the lethal effects of endotoxins. Beeson[5] has postulated that the blockade permitted the endotoxin to circulate in the blood longer and thereby allowed it to reach and damage susceptible tissues more readily, instead of being sequestered in the elements of the reticuloendothelial system. These results might also be interpreted to mean that interference with the normal breakdown of the endotoxin simply permitted the toxic material to build up to lethal amounts more readily. The latter explanation would appear more in harmony with the data presented here.

In patients inadvertently transfused with contaminated blood, death may result either from the total number of bacteria injected or from sublethal numbers of organisms which are able to multiply rapidly and liberate a lethal amount of endotoxin. In the former case, antibiotics would be of little value in preventing death, since a lethal amount of endotoxin would already have been infused. Antibiotics, by preventing bacterial multiplication from reaching toxic levels, would be of great potential benefit in the latter case, provided they were administered within 1 hour of the transfusion. Because of the difficulty in recognizing early the syndrome resulting from the transfusion of contaminated blood, treatment sometimes has been delayed. Under these conditions, antibiotics cannot be expected to prevent death, since a lethal amount of endotoxin might already have been accumulated.[7, 8] Therefore, in the advent that a blood transfusion results in the appearance of early signs*

*For an excellent description of the syndrome resulting from the transfusion of contaminated blood, see Braude et al.: Arch. Int. Med. 92: 75-84, 1953, or reference (7).

suggesting contaminated blood has been infused, massive amounts of antibiotics covering all probable types of bacterial contaminants should be given without delay.

SUMMARY

The intraperitoneal injection of 10^9 living coliform bacteria in mice produced massive bacteremia which remained constant until all animals died 5 or 6 hours later.

The injection of 10^8 bacteria resulted in a peak of bacteremia in 1 hour, but thereafter the number of organisms circulating in the blood rapidly declined and by the twelfth hour the blood was sterile. The majority of these animals survived.

The apparent relationship between the inability of the reticuloendothelial system to remove huge numbers of bacteria from the blood and death of the animal may be misleading, since it was clearly shown that intensive single or combined antibiotic therapy begun 3 hours after the bacteria were injected promptly suppressed the bacteremia, but nonetheless the majority of animals died. Antibiotics administered within 1 hour after the bacteria were much more effective in preventing death.

It was suggested that death following the injection of lethal numbers of coliform bacteria might be due to sufficient bacterial multiplication to result in the rapid accumulation of a lethal dose of endotoxin. Prompt antibiotic therapy, by suppressing bacterial multiplication, would favor the rapid destruction of the organisms and prevent the endotoxin from reaching a lethal concentration.

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LABORATORY METHODS

THE DETERMINATION OF CALCIUM IN BIOLOGIC MATERIAL

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INTRODUCTION

CHEMICAL procedures for the quantitative determination of calcium in biologic material have been based on (1) precipitation of the calcium from solution as its insoluble oxalate, or phosphate then (2) analyzing for the precipitate anion, after purification, to give an indirect measurement of calcium,[1-5] or (3) analyzing the precipitate directly for the element.[6, 7] These indirect isolation procedures are time-consuming and require a high degree of analytic skill for accurate results.

Within the past few years methods for the direct determination of calcium in solution have been developed. Direct measurements were carried out by water laboratories using titrimetric methods[10, 11] based on the work of Schwarzenbach and associates[8, 9] on the formation of complexes of alkaline earth metals with disodium dihydrogen ethylenediamine tetraacetate (EDTA).

Sobel and Hanok[12] developed an indirect procedure for serum calcium employing EDTA reagent for titration and the dye Eriochrome black T as an indicator. Since then other procedures using these reagents have appeared in the literature. Unfortunately the resulting values give total magnesium plus calcium and necessitate an analysis for magnesium in order to calculate calcium. Greenblatt and Hartman,[13] Buckley and co-workers,[14] and Elliott[15] developed visual titration methods for serum using ammonium purpurate as the indicator for calcium. The visual end point of this indicator in serum, however, is indistinct and easily obscured by hemolysis and bilirubinemia. To overcome these difficulties Kibrick and associates[16] and Fales[17] adapted the EDTA-purpurate procedure for serum to photometric measurement.

The method of Kibrick and co-workers in our laboratory has not proved sufficiently accurate for our purpose due to the lack of a sharp titrimetric end point. Fales's procedure, on the other hand, is sufficiently accurate but is tedious.

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The greatest deterrant to the use of EDTA-purpurate titration procedures for calcium has been the interference encountered when specimens of high phosphate content, such as urine and feces, are to be analyzed. This has restricted their use to serum analysis.

The method to be described has overcome the tediousness of a photometric titration, is simple, rapid, and accurate, and can be used for the determination of calcium in serum, urine, feces, and diets.

MATERIALS AND METHODS

Reagents and Equipment.—

1. Disodium dihydrogen ethylenediamine tetraacetate.*
   Stock solution—3.6 Gm. EDTA is dissolved in a few milliliters of distilled water and the solution is made up to 1 L.
   Working solution—25 ml. of the stock solution is diluted to 500 ml. with water. One milliliter of this solution is equal to approximately 0.02 mg. of calcium.
2. Ammonium purpurate.†
   Murexide, 0.090 Gm. is dissolved in 500 ml. of distilled water. Care must be taken that the indicator is completely in solution since this concentration is optimal for the titration. A freshly prepared solution when read against a water blank at 620 mμ should give an optical density reading of roughly 0.40 to 0.43.
3. Indicator—EDTA titrating solution.
   Exactly equal quantities of the working EDTA solution and the murexide solution are combined and mixed well.
4. Sample indicator diluting solution.
   This is the murexide solution prepared in Paragraph 2.
5. Sodium hydroxide 1.5N.
6. Evelyn photometer-galvanometer scale calibrated in optical density or per cent transmission.
7. Automatic pipette adjusted to deliver 1.0 ml.
   A drawing of the titration assembly used in our laboratory is given in Fig. 1. This machine employs the principle of intermittent air mixing. Aside from the initial expense of the automatic pipette (Fisher Volustat), this assembly is inexpensive, easy to construct, and greatly accelerates the titration procedure.
8. Standard calcium solution, 10 mg. per 100 ml. Prepared from reagent grade calcium carbonate. The concentration is checked by the standard oxalate-permanganate procedure.
9. Sodium tungstate $Na_2WO_4 \cdot 2H_2O$, reagent grade, 66 per cent.
10. Morpholine nitrate,‡ 48 per cent.
    Place 28 ml. of purified morpholine in a flask. Cool in an ice bath and slowly add nitric acid (1 part concentrated acid to 1 part water) until the solution is neutral or slightly acidic (about 40 to 45 ml. required). Dilute to 100 ml. with distilled water.
11. Morpholine nitrate–nitric acid reagent.
    Take equal portions of morpholine nitrate solution and nitric acid (1 part concentrated acid to 3 parts water) and mix well.

Procedure.—

Serum: Into a clean Evelyn cuvette pipette 1 ml. of serum, 1 ml. of 1.5N NaOH, and 2 ml. of the sample indicator diluting solution.‖ The contents of the tube are mixed and

*I am indebted to the Bersworth Chemical Company, Framingham, Massachusetts, for furnishing the reagent.

†Murexide, C. P. obtained from the Fisher Scientific Company.

‡Morpholine, purified, obtained from the Fisher Scientific Company.

‖The use of burettes to add reagents will expedite matters.

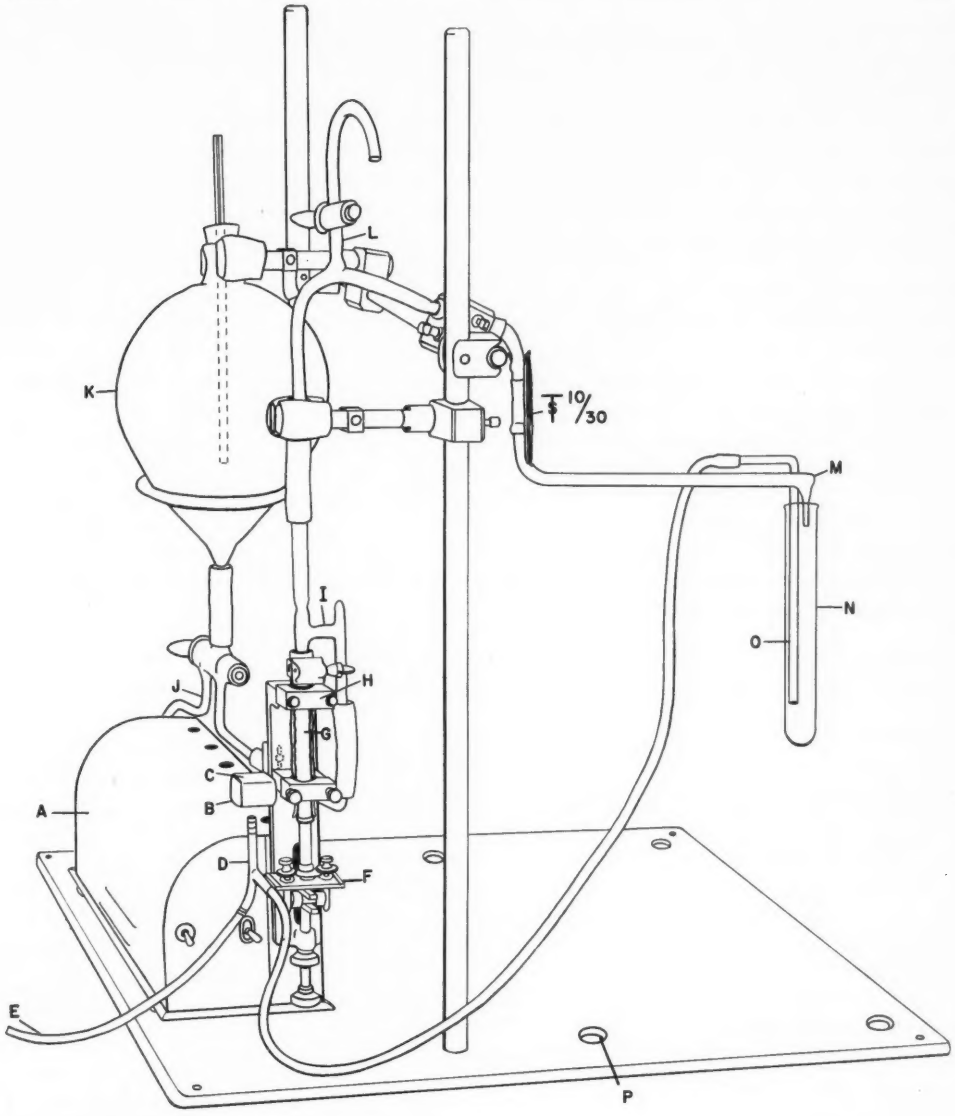

Fig. 1.—Titration assembly.

A, Fisher Volustat—Fisher Scientific Company.

B, Sponge-rubber cushion backed by foam rubber and cemented to the top of housing (*C*).

C, Adjustable housing for rubber cushion.

D, T tube, brass, intermittent air leak.

E, Tube from compressed air source.

F, Syringe barrel clamp with T tube (*D*) attached.

G, 3 ml. syringe.

H, Plastic clamps for anchoring syringe. Volustat comes equipped with one.

I, Glass valves of the Fisher Volustat.

J, Drain for reservoir (*K*).

K, 1,000 ml. leveling bulb reservoir.

L, Outlet to remove entrapped air.

M, Movable ejector—7 mm. giass tubing.

N, Evelyn Colorimeter cuvette.

O, Air stirrer 4 mm. glass tubing.

P, Holes for mounting Evelyn colorimeter.

read in the photometer at a wave length of 620 mμ, aperture 6 ml. For extremely low calcium concentrations use 2 ml. of serum, 2 ml. of hydroxide, and 4 ml. of the sample indicator diluting solution.

With the automatic pipette regulated to deliver 1 ml. of standard indicator–EDTA titrating solution, 1 ml. is pipetted into the tube. The contents of the tube are mixed and a reading taken (with the assembly described here, mixing is automatic). The procedure is continued until the end point is reached, as can be noted by no further change in the galvanometer reading. The end point is determined graphically as the intercept of the linear portion of the titration curve with the end-point plateau as described by Fales (if per cent T is used, plot on semilogarithmic paper, with O.D. use graph paper).* One milliliter of standard calcium solution is also titrated as previously to determine the exact equivalence of the EDTA.

Urine or ashed biologic specimens†: Pipette 4 ml. of sample into a 15 ml. glass-stoppered centrifuge tube. Add 2 ml. of tungstate reagent from a burette and 4 ml. of morpholine nitrate–nitric acid reagent and mix well. An immediate precipitation of both morpholine phosphotungstate and morpholine tungstate will be noted. Allow the tubes to stand for 1 hour to ensure complete precipitation. Centrifuge for several minutes. Pipette 1 ml. of the clear supernatant liquid into an Evelyn tube, 1 ml. of 1.5 N NaOH,‡ and 2 ml. of the sample indicator diluting solution (if larger aliquots are necessary simply use more hydroxide and indicator solution in multiples of the proportions given previously). Proceed as described for serum titration.

Calculations

$$\text{Factor} = \frac{\text{mg. Ca/ml. of standard}}{\text{ml. EDTA to titrate 1 ml. of standard}}$$

$$\text{Serum direct: } \frac{(\text{ml. EDTA})\ (\text{F})\ (100)}{\text{ml. titrated}} = \text{mg. Ca/100 ml.}$$

Serum or urine freed of phosphate:

$$\frac{(\text{ml. EDTA})\ (\text{F})}{\text{ml. titrated}} \times \frac{10}{\text{ml. sample pipetted}} \times \text{total volume} = \text{mg. Ca total}$$

Digests:

$$\frac{(\text{ml. EDTA})\ (\text{F})}{\text{ml. titrated}} \times \frac{(10)\ (\text{volume digest solution})}{\text{ml. sample pipetted}} \times \frac{\text{total weight}}{\text{aliquot weight}} = \text{mg. Ca total}$$

RESULTS AND DISCUSSION

Typical results depicted in Tables I, II, and III make it readily apparent that this procedure compares very favorably with that of the standard oxalate-permanganate method. The recovery of calcium added to urine, or diet, and fecal ash is quantitative.

*At least three points in a straight line should be obtained on the steep portion of the titration curve, preferably more. The curve tapers at both ends. Points in the tapering sections must not be used in plotting.

†Urine may be preserved by adding 10 ml. of glacial acetic acid to the 24-hour specimen. This prevents the precipitation of calcium. In our laboratory, feces, diets, tissues, etc. are ashed with nitric and perchloric acids.[18] After evaporation of the acid the ash is dissolved and diluted to the desired volume with distilled water.

‡On the addition of alkali (1.5N) to the treated urine just prior to titration a transient blue coloration is occasionally observed. This disappears in a matter of minutes and does not interfere with the titration.

Kibrick and associates and Fales made a significant contribution to the use of purpurate as an indicator for EDTA titration when they developed procedures for the photometric titration of calcium. Many of the features of Fales's titration method have been incorporated in this determination.

The principle of "internally blanking" a photometric titration in order to eliminate the titration of a blank is to our knowledge entirely new. It consists essentially of maintaining the concentration of the indicator in the sample

TABLE I. RECOVERY OF ADDED CALCIUM AND COMPARISON OF THIS PROCEDURE WITH THE STANDARD OXALATE-PERMANGANATE METHOD FOR CALCIUM

	CALCIUM				
SAMPLE	PRESENT (GM./100 ML. [GM.])	ADDED (GM./100 ML. [GM.])	TOTAL (GM./100 ML. [GM.])	RECOVERED (GM./100 ML. [GM.])	CLARK-COLLIP (GM./100 ML. [GM.])
Diets	0.019	0.032	0.052	0.033	0.019
	0.042	0.085	0.128	0.086	
	0.193	0.170	0.368	0.175	
	0.332	0.605	0.910	0.578	0.332
	0.673	5.334	6.041	5.368	0.667
	0.811	5.571	6.563	5.752	0.817
Feces	0.286	0.482	0.761	0.475	
	0.533	0.463	0.979	0.446	
	1.526	0.582	2.108	0.582	1.527
	2.184	2.282	4.393	2.209	2.186
	2.928	2.431	5.307	2.379	2.850
	10.643				10.631
Urine	0.0143				0.0141
	0.0228	0.0303	0.0531	0.0303	0.0231
	0.0255	0.0303	0.0551	0.0296	0.0251
	0.0320	0.0368	0.0683	0.0363	0.0327
	0.0375	0.0368	0.0733	0.0358	0.0379
	0.0495	0.0368	0.0856	0.0361	0.0506

TABLE II. COMPARISON OF THE DIRECT TITRATION OF SERUM WITH THE STANDARD OXALATE-PERMANGANATE METHOD FOR CALCIUM

CALCIUM*	
DIRECT TITRATION (MG./100 ML.)	CLARK-COLLIP (MG./100 ML.)
6.6	6.6
9.5	9.5
9.7	9.5
11.5	11.7
15.6	15.6†

*Duplicate determinations.
†Serum bilirubin 41 mg. per 100 ml.

TABLE III. COMPARISON OF THE DIRECT TITRATION OF SERUM WITH TITRATION AFTER THE REMOVAL OF PHOSPHATE

CALCIUM*	
DIRECT TITRATION (MG./100 ML.)	PHOSPHATE FREE (MG./100 ML.)
9.0	9.0
9.3	9.3
10.0	10.0
10.3	10.3

*Duplicate determinations.

and the titrating solution equal. Since the photometer measures only the concentration of an absorbent, the end-point plateau is realized without the necessity of employing a blank and the dilution effect of the titrating fluid is nullified. It is extremely advantageous when one is confronted with an unstable indicator such as ammonium purpurate. The principle may well be applied to other photometric titrations.

The titration assembly described employs automatic intermittent air flow as an efficient means of mixing. This has the distinct advantage of eliminating the fluctuations in light transmission inherent in methods employing continuous gas mixing.

As mentioned in the introduction, the phosphate ion is responsible for the marked inhibition of the purpurate color change when calcium-containing samples of moderate or high phosphate content are titrated with EDTA and purpurate. The procedure described for the removal of this interfering ion makes the direct determination of calcium in biologic material possible. Classical procedures for the removal of phosphate are of little value since the reagents used interfere with the determination. Ion exchange resins can be used in column procedures, but these are time-consuming. The borate salt of Amberlite I R 400 is excellent for this purpose. The formation of the heteropolyacid, phosphotungstic, followed by precipitation with morpholine is a more practical procedure. Although several other precipitants of phosphotungstic acid are available, morpholine has been found to be superior. The reagent is inexpensive, and its solubility properties well suited to this procedure. Morpholine will precipitate phosphotungstic acid quantitatively from solution. It has the added advantage of possessing an insoluble tungstate. Thus, the supernatant fluid after the addition of morpholine is essentially free of both tungstic and phosphotungstic acids.

Ordinarily, serum specimens are not freed of phosphate ion but are titrated directly. This possible because of the low phosphate concentration normally present in serum. In severe renal disease and after phosphate infusions, interfering serum concentrations of phosphate may be present. Serums having phosphorus concentrations exceeding 10 mg. per 100 ml. should be freed of phosphate prior to titration.

Interference from the magesium ion is not a problem. Under the conditions of this determination, magnesium concentrations in urine or digests must exceed 80 to 100 mg. per 100 ml. before interference occurs. Ferric ions likewise present no difficulty. Concentrations of 6 to 10 mg. per 100 ml. of urine or digests cause no interference. Other cations and anions occurring in biologic material are without effect.

SUMMARY

A rapid, simple, and accurate procedure for the determination of calcium in biologic material has been described.

A principle of internally blanking a photometric titration and an automatic titrating assembly employing intermittent air agitation have been presented.

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A GRAPHIC METHOD FOR THE CONVERSION OF TRANSMISSION CURVES TO A CORRECTED DENSITY CURVE FOR THE CALCULATION OF PROTEINS SEPARATED BY PAPER ELECTROPHORESIS*

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MANY methods have been used for the quantitation of plasma proteins separated by paper electrophoresis. The procedure should be relatively simple, give reproducible results, and provide a graphic record so that a visual comparison of normal and abnormal patterns may be made. The more commonly used methods are those which depend upon the measurement of the density of the dye-stained protein at intervals along the paper strip, either by measuring in a spectrophotometer the density of the dye eluted from segments of the stained paper[1,2] or by scanning the dyed strip with a densitometer.[3,4] A plot of the densities obtained by either method gives a curve similar in contour to the classical Tiselius pattern. The distribution of the major protein fractions does not agree, however, when calculated by the two procedures because the density of the dye in the dry paper does not follow Beer's law throughout the range of concentrations usually encountered.[5] A correction must therefore be applied to the curve obtained by direct scanning if the values obtained from this curve are to be brought into agreement with those obtained from elution data. In order to obtain a graphic record without plotting a large number of points, a recorder may be used in conjunction with the densitometer. Although a recorder is now available that integrates the curve, the usual commercial recorders graph transmission which must be converted to density before planimetry may be used for quantitation.

In order to facilitate the conversion of a transmission curve to a density curve corrected for deviations of the dye from Beer's law, a double-scale rule was found useful. The construction of this rule is presented together with results obtained from the corrected density curves and from the density of the dye eluted from segments of a dyed strip.[6]

METHODS

An "optical wedge" was prepared in order to determine the correction factors necessary to adjust the apparent densities of the dry strip to corrected densities.[5] A long strip of Whatman No. 1 filter paper was slowly drawn through a solution of bromphenol blue in 2 per cent acetic acid while the dye was gradually being diluted with the dilute acetic acid. The density along the dried strip was graphed by a Brown recorder con-

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nected to a densitometer. The transmissions, which varied from 90 per cent to 10 per cent, were converted to the corresponding densities. Segments of equal width representing densities from 0.3 to 1.9 were cut from the strip, eluted with 5 per cent sodium carbonate in 50 per cent methyl alcohol, and read at 590 millimicrons in a Beckman B spectrophotometer. The density of the eluted dye in each segment was multiplied by the ratio of the density of the dye in segment 1 (calculated from transmission) to the density of the eluted dye in the same segment. The density of the eluted dye is then numerically equivalent to the corrected density for each of the strips. These are tabulated in Table I.

TABLE I. A COMPARISON OF THE APPARENT DENSITY VALUES AND CORRECTED DENSITY VALUES OF SEGMENTS OF THE "OPTICAL WEDGE"

SEGMENT NUMBER	APPARENT DENSITY	CORRECTED DENSITY
1	0.345	0.345
2	0.500	0.581
3	0.680	0.850
4	0.925	1.200
5	1.05	1.57
6	1.45	3.70
7	1.90	9.7

CONSTRUCTION OF DOUBLE-SCALE RULE

In order to facilitate the construction of a corrected density curve from a transmission curve, a double-scale rule was found essential. This was constructed from thin plastic in the following manner:

The percentage transmission was plotted as the ordinate on the recorder paper against the corresponding density on the abscissa. The density on the abscissa was then plotted against "corrected" density on the ordinate (Table I). The linear unit for "corrected" density on the ordinate was so chosen that the corrected density curve would lie under the transmission curve. Each point on the lower curve represents the corrected density for the transmission directly above it on the graph. The double curve is shown in Fig. 1.

The curves were then transferred to a square sheet of thin plastic with the 100 per cent transmission at the base of the sheet. The upper curve was scratched into the plastic. The plastic was cut through along the lower curve down to about the level of the 95 per cent transmission and a narrow segment of plastic was cut away below the curve. This allowed a mark to be made on the paper beneath the plastic.

USE OF DOUBLE RULE

The double rule was used to convert twenty transmission curves to corrected density curves. The distribution of the protein in various fractions obtained by planimetry of the latter was compared with that obtained by the measurement of the dye eluted from the paper strip.

The Durrum procedures[4] were used for the separation and dying of the protein on the strips. Each strip was run through the densitometer and the curve obtained on a Brown recorder. This curve was fastened to a drawing board with the base line against a fixed straight edge. The double plastic rule was placed over the graph with the bottom against this edge and then moved horizontally until the upper curve (transmission) touched a maximum point on the graph. The corresponding point on the lower curve was then marked on the recorder paper. Other maxina, minima, and a few intermediate points were marked in the same manner and the corrected density curve drawn as shown in Fig. 2.

The distribution of the proteins between the various fractions was obtained by planimetry. The strips were then cut into the segments which represented the major pro-

tein fractions. The transmission curve was used to select the points of separation. These segments were eluted with 5 per cent sodium carbonate in 50 per cent methyl alcohol and the eluates read at 590 millimicrons in a Beckman B spectrophotometer. The distribution of the protein in the various fractions was calculated. A comparison of the results obtained by the two methods is presented in Table II.

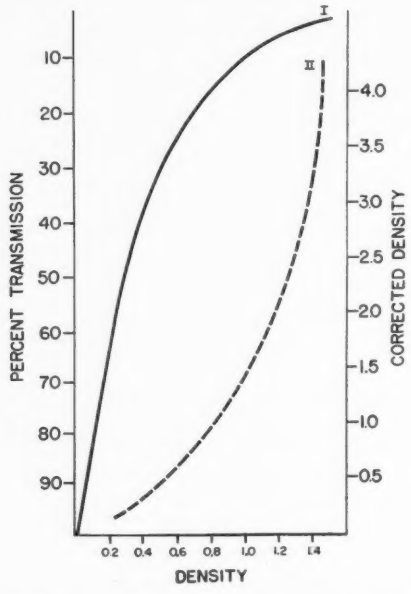

Fig. 1.—The double curve from which the plastic double-scale rule is constructed.

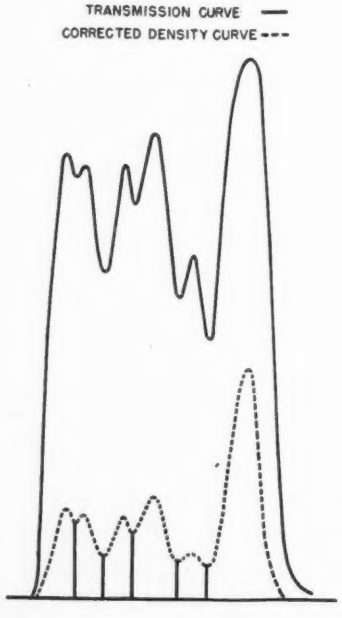

Fig. 2.—A transmission curve with the corresponding density curve.

TABLE II. A COMPARISON OF THE RESULTS OBTAINED FROM THE CORRECTED DENSITY (D) AND ELUTION DATA (E)

		ALBUMIN		GLOBULINS									
				ALPHA-1		ALPHA-2		BETA-1		BETA-2		GAMMA	
		%	GM.	%	GM.	%	GM.	%	GM.	%	GM.	%	GM.
1	d	62.4	3.68	4.9	0.29	9.4	0.55	11.3	0.66	5.3	0.31	6.6	0.39
	e	62.3	3.66	4.9	0.29	9.6	0.57	11.6	0.69	4.9	0.29	6.7	0.39
2	d	60.2	4.45	5.4	0.40	10.5	0.78	10.5	0.78	4.1	0.30	9.3	0.69
	e	60.5	4.48	5.5	0.41	11.0	0.81	11.0	0.82	4.3	0.32	7.5	0.46
3	d	63.0	4.91	4.7	0.37	12.3	0.96	10.0	0.83	4.2	0.33	5.1	0.40
	e	63.8	4.97	5.3	0.42	13.5	1.05	8.5	0.66	4.7	0.37	4.2	0.33
4	d	64.4	2.98	6.3	0.29	11.2	0.52	8.4	0.39	3.5	0.16	6.3	0.29
	e	69.7	3.20	5.9	0.27	9.6	0.44	7.4	0.34	3.0	0.14	4.4	0.21
5	d	54.2	3.74	8.0	0.55	10.6	0.73	13.3	0.92	7.3	0.31	6.6	0.46
	e	54.0	3.72	6.6	0.46	9.5	0.66	17.6	1.21	5.9	0.41	6.4	0.44
6	d	58.0	4.35	5.0	0.37	6.8	0.41	12.9	0.97	9.1	0.68	8.2	0.62
	e	59.4	4.46	4.2	0.32	5.6	0.42	10.7	0.80	8.8	0.66	9.7	0.73
7	d	40.6	2.72	6.3	0.42	14.9	1.00	18.1	1.21	—	—	20.1	1.35
	e	45.1	3.02	5.1	0.34	12.4	0.83	16.7	1.12	—	—	20.7	1.39
8	d	53.5	3.53	7.2	0.48	6.1	0.41	11.4	0.75	9.6	0.63	12.2	0.80
	e	54.7	3.62	6.7	0.44	6.7	0.44	10.8	0.71	9.0	0.59	12.2	0.80
9	d	53.4	3.21	7.5	0.47	10.0	0.60	10.7	0.64	11.3	0.68	7.1	0.43
	e	54.8	3.28	7.7	0.47	10.3	0.62	9.8	0.59	9.3	0.56	8.0	0.48
10	d	36.2	2.46	6.5	0.44	13.6	0.93	16.2	1.10	10.3	0.70	17.2	1.17
	e	35.7	2.43	6.4	0.43	12.8	0.87	16.4	1.12	10.3	0.70	18.4	1.25
11	d	61.6	4.45	6.1	0.44	11.6	0.83	10.6	0.75	4.8	0.34	5.3	0.38
	e	63.2	4.55	6.1	0.44	10.2	0.73	10.8	0.78	4.5	0.32	5.2	0.37
12	d	50.0	2.31	6.8	0.31	13.2	0.61	13.8	0.63	8.1	0.37	8.1	0.37
	e	56.0	2.58	6.5	0.30	12.0	0.55	8.9	0.41	7.2	0.33	9.4	0.43
13	d	47.8	3.50	7.6	0.55	12.9	0.91	15.8	1.15	6.0	0.44	10.3	0.75
	e	49.1	3.59	5.4	0.39	12.5	0.91	13.1	0.96	9.7	0.71	10.2	0.74
14	d	30.6	1.93	9.1	0.57	11.4	0.72	11.7	0.73	24.2	1.52	13.1	0.83
	e	30.9	1.95	7.4	0.47	11.6	0.73	11.2	0.71	24.4	1.54	14.5	0.91
15	d	43.2	2.81	7.2	0.47	14.7	0.96	18.0	1.17	5.0	0.33	11.9	0.77
	e	43.4	2.86	7.1	0.46	15.2	0.99	18.1	1.18	4.6	0.30	11.6	0.75
16	d	58.4	3.04	8.7	0.45	6.4	0.33	8.4	0.46	13.4	0.70	4.3	0.22
	e	60.0	3.12	7.9	0.41	6.4	0.33	7.6	0.40	12.9	0.67	4.7	0.24
17	d	54.3	3.58	6.6	0.44	9.1	0.60	9.9	0.65	5.8	0.38	14.3	0.95
	e	54.4	3.59	6.6	0.44	8.8	0.61	9.3	0.61	5.7	0.38	15.2	1.00
18	d	40.0	2.40	6.7	0.40	11.6	0.70	12.4	0.74	9.6	0.58	19.7	1.18
	e	40.5	2.43	5.1	0.31	11.0	0.66	11.2	0.67	10.7	0.64	21.5	1.29
19	d	51.3	2.87	8.4	0.47	8.4	0.47	11.4	0.64	12.3	0.69	8.9	0.46
	e	51.1	2.86	8.6	0.48	8.0	0.45	11.0	0.52	12.4	0.69	8.9	0.46
20	d	44.1	2.56	10.0	0.58	10.2	0.59	10.9	0.59	14.8	0.86	10.0	0.58
	e	44.0	2.55	8.8	0.51	10.7	0.62	10.7	0.62	15.8	0.92	10.0	0.58
	d	Mean	3.32		0.44		0.69		0.78		0.55		0.65
		S. D.	±.66		±.06		±.22		±.21		±.31		±.32
	e	Mean	3.34		0.40		0.66		0.76		0.55		0.66
		S. D.	±.67		±.14		±.27		±.24		±.31		±.34
	$t_{(19)}$	= 3.5		t = 3.5		t = 1.7		t = 0.8		t = 1.7		t = 0.5	

TABLE III. A COMPARISON OF RESULTS OF REPLICATE DETERMINATION OF SERUM PROTEIN

SERUM	NUMBER OF STRIPS		ALBUMIN (GM.)	GLOBULIN				
				ALPHA-1 (GM.)	ALPHA-2 (GM.)	BETA-1 (GM.)	BETA-2 (GM.)	GAMMA (GM.)
A	6	Mean	2.77	0.36	0.94	1.12	0.90	2.69
		S. D.	±0.05	±0.02	±0.01	±0.05	±0.01	±0.04
B	9	Mean	4.48	0.38	0.82	1.31	0.94	1.86
		S. D.	±0.04	±0.01	±0.02	±0.05	±0.04	±0.03

To establish the reproducibility of the results obtained by this procedure, six strips were prepared from one serum and nine from a second serum. Two electrophoresis units were used and the strips were divided into two runs. The protein was quantitated according to the procedure previously outlined. The mean and standard deviation of the results are tabulated in Table III.

DISCUSSION

The "Student" t values calculated for the paired data indicated that the differences between the two methods did not vary significantly from zero for alpha-2, beta-1, beta-2, and gamma globulins. The graphic method gave slightly lower values for albumin and slightly higher values for alpha-1 globulin than did the elution procedure. The differences were not of such magnitude so as to affect the interpretation of results. Results on replicate determinations show a satisfactory degree of agreement.

SUMMARY

1. The construction of a double rule to facilitate the conversion of a transmission graph to a density curve corrected for deviation from Beer's law is outlined.

2. A comparison of the results obtained by using the double rule and by elution of the dye representing the major protein fractions in the paper strip with the subsequent measurement of the density of dye shows satisfactory correlation between these methods of calculation.

3. Good agreement is obtained between results calculated from corrected density curves of replicate runs of the same serum.

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STUDIES ON PRIMARY AMINES

III. Limiting and Optimum Conditions for Quantitative Determination by the Ninhydrin Method

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QUANTITATION of biologic primary amines as a class of compounds has been inaccurate, insensitive, and nonspecific. An improved technique for the estimation of some of them in dilute solution was recently outlined.[1] Here the limiting and optimum conditions of this method are reported in detail. In brief, assay depends on the formation of a colored complex when amine and ninhydrin are heated in anhydrous alcoholic pyridine.[2]

METHOD

To primary amine† in water-free isopropanol are added pyridine and ninhydrin (triketohydrindene hydrate) so that their concentrations are, respectively, 50 and 0.04 per cent (by weight). Without delay the mixture is heated for seven minutes at 87° C. and then rapidly cooled to 20° C. The optical density is read at 417 mμ on the Beckman photoelectric spectrophotometer (band width 4 mμ) after 15 to 90 minutes at room temperature.‡

RESULTS

Since at least three reactants and perhaps more than one product are involved, it is difficult to define precisely the series of reactions which presumably occurs. At high concentrations, first of amine, then of ninhydrin, the reaction rate was appreciable at 30° C.; under these conditions the rate-limiting step was first order with respect to amine and second order with respect to ninhydrin (Table I), suggesting that the nitrogen of the primary amine bound two ninhydrin molecules, as has been previously postulated for amino acids in water.[3] Consistent with this thesis was the observation that under suitable conditions ninhydrin formed a brown solution with concentrated ammonia, whereas it did not visibly react with secondary or tertiary amines (Table II). The function of pyridine in the reaction is uncertain; it may react with ninhydrin initially, since preincubation of pyridine and ninhydrin, but not of pyridine and amine, shortened the marked lag in attaining the maximal rate of color formation (Fig. 1). The result was a greater initial optical density under given conditions, although there was no change in the eventual maximal rate of opacity increase.

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†Many of the amines were supplied through the generosity of the Monsanto Chemical Company, St. Louis. Commercially available amines were also obtained from Eastman Kodak Company, Rochester, New York. Both anhydrous bases and hydrochlorides were tested.

‡In order to study the reaction between ninhydrin and primary amine under varying conditions, the method previously reported[1] was modified by the elimination of acid from the isopropanol solvent.

TABLE I. MAXIMUM RATE OF COMPLEX FORMATION* AS A FUNCTION OF REACTANT CONCENTRATION

ISOAMYLAMINE MILLIMOLARITY	NINHYDRIN MILLIMOLARITY	MAXIMAL INCREASE IN OPTICAL DENSITY PER MINUTE
3.33	10.0	0.0990
1.67	10.0	0.0527
0.833	10.0	0.0301
0.467	10.0	0.0151
3.33	10.0	0.101
3.33	5.00	0.0281
3.33	2.50	0.00595

*As measured by the change in 417 mμ optical density, the solvent being one half pyridine and one half isopropanol and the temperature 30° C. An initial lag period preceded an interval when the rate of increase in optical density was constant and maximal. This interval lasted from five minutes to one hour. All readings were made before there was an appreciable diminution in reactant concentration.

TABLE II. MOLECULAR EXTINCTION COEFFICIENTS AT 417 mμ FOR THE NINHYDRIN COMPLEX OF VARIOUS AMINES

AMINE	POSSIBLE PARENT AMINO ACID	MOLECULAR EXTINCTION COEFFICIENT BASE	
		1ST VALUE	REPEAT
	Part 1		
Ammonia		10^2	
Methylamine		6×10^3	
Dimethylamine		< 10	
Trimethylamine		< 10	
Tetramethylammonium chloride		< 10	
	Part 2		
Methylamine	Glycine	5,620	5,710
Ethylamine	Alanine	5,550	5,820
n-Propylamine	Glutamic acid	5,860	5,930
n-Butylamine	Norvaline	5,800	5,880
n-Amylamine	Norleucine	5,610	5,780
n-Hexylamine		6,090	6,010
n-Heptylamine		6,050	5,930
n-Decylamine		5,600	5,830
n-Octadecylamine		5,530	5,690
Isopropylamine		1,680	1,800
Isobutylamine	Valine	4,670	4,860
Isoamylamine	Leucine	5,800	5,820
Ethanolamine	Serine	5,120	5,200
Histamine	Histidine	3,050	3,150
Histamine hydrochloride		3,350	3,480
Tyramine hydrochloride	Tyrosine	3,380	3,250
Tryptamine hydrochloride	Tryptophan	4,800	5,020
β-Phenylethylamine	Phenylalanine	3,500 ±	1,000

One micromol of primary amine plus 4 mg. of ninhydrin in 10 ml. of a half-and-half mixture of pyridine and isopropanol were heated to 87° C., cooled, and the optical density determined at 417 mμ. The resultant reading was multiplied by 10,000 to yield a figure corresponding to the molecular extinction coefficient.

The visible absorption spectra of ninhydrin-amine complexes fell into two distinct categories (Fig. 2). That of isoamylamine illustrates the double-peaked type characteristic of aliphatic amines. That of tryptamine represents the hyperboloid type associated with aromatically and heterocyclically substituted amines. The former were blue and the latter brown. After coloring it was possible to separate these two groups of compounds by their distribution between water and nonpolar solvents. The blue aliphatic amine complexes were hydrophilic with water-chloroform partition coefficients of 10 ± 2, whereas

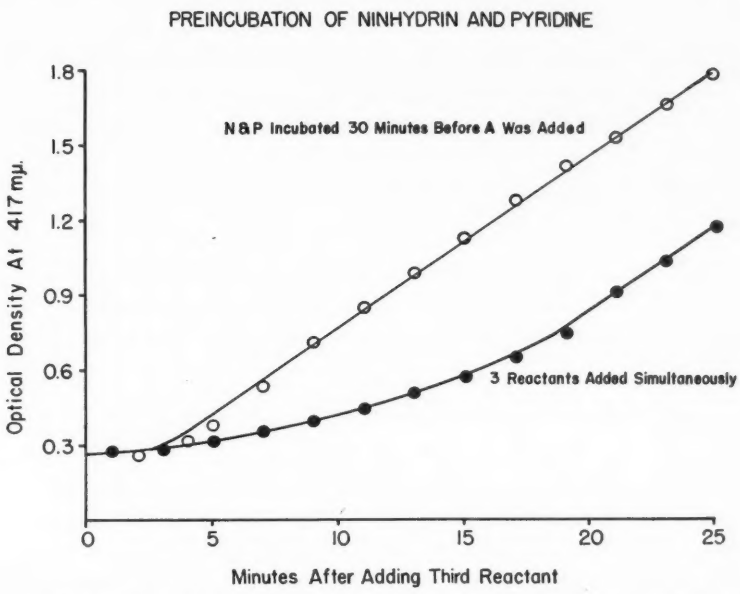

Fig. 1.—The shortened lag in attaining the maximal reaction rate following preincubation of ninhydrin and pyridine. The entire procedure was done at 30° C. In each case the final concentration of amine was 20 mM., that of ninhydrin was 10 mM. (0.175 per cent), and that of pyridine 50 per cent, isopropanol being the remaining constituent in the reaction mixture. The open dots illustrate an experiment when ninhydrin and pyridine were mixed 30 minutes prior to the addition of isoamylamine. Time on the abscissa is measured from the addition of the amine (*A*); three minutes thereafter the maximum rate of color formation was attained. The solid dots represent the usual situation when ninhydrin, pyridine, and amine were added simultaneously. For more than 15 minutes there was a submaximal rate of color increase, although thereafter the two curves were parallel. When ninhydrin and amine or pyridine and amine were incubated together, the time-opacity curve after the addition of the third reactant was very similar to that indicated by the solid dots. A ninhydrin-pyridine-isopropanol blank was 0.25 due to the high concentration of the ninhydrin.

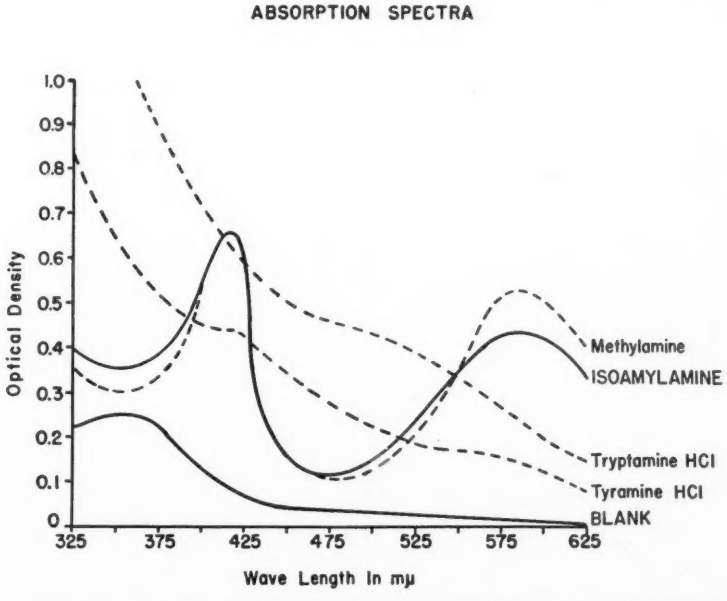

Fig. 2.—Absorption spectra of the ninhydrin-isoamylamine complex and for the amineless blank are shown by solid lines. The absorption spectrum for the ninhydrin complex of another aliphatic amine base and of an aromatically and a heterocyclically substituted amine hydrochloride are indicated by broken lines. The final concentration of each amine was 100 micromolar. The hydrochlorides of isoamylamine and methylamine gave the same double-peaked curve as the free bases.

their brown counterparts were hydrophobic with coefficients of 0.1 ± 0.02. The former could be converted to a form indistinguishable by absorption spectrum or partition coefficient from the latter. This conversion was brought about in several ways (Fig. 3), one of which was by sufficient increase in the reaction time or temperature, suggesting that the blue complex might be an intermediate in the formation of the brown.

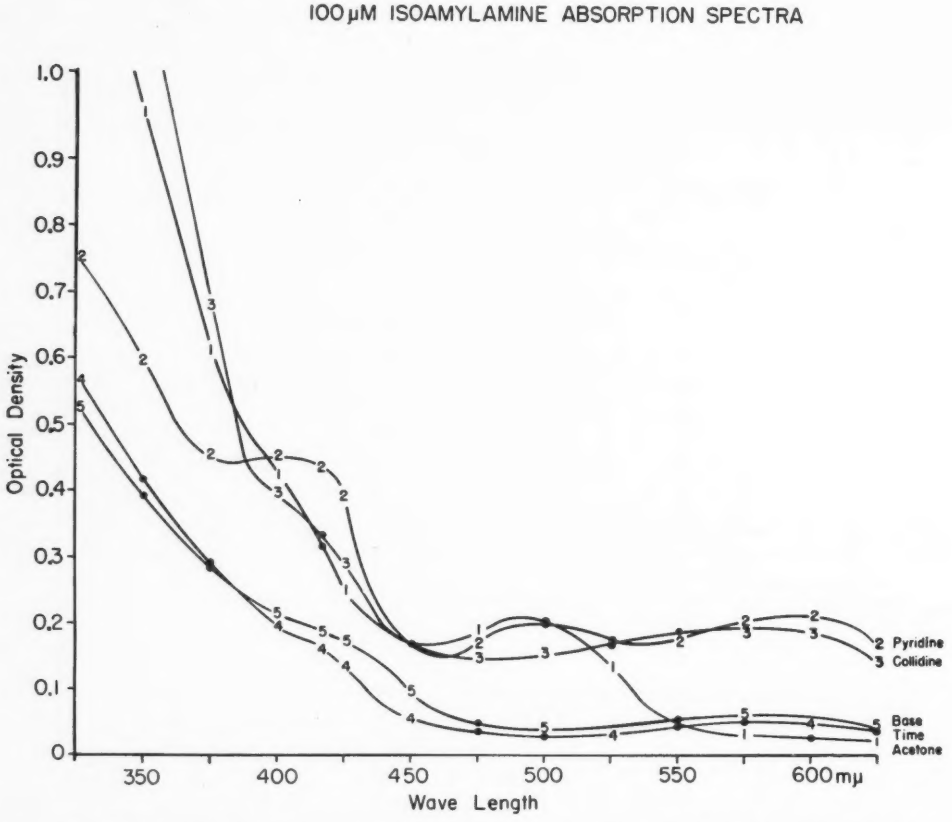

Fig. 3.—Absorption spectra of the ninhydrin-amine complex for 100 micromolar isoamylamine solutions converted by various means from the typical double-peaked aliphatic amine curve to hyperboloid forms similar to those seen with aromatically or heterocyclically substituted amines. For Curve 1 isopropanol was replaced by acetone. For Curve 2 the usual clear pyridine was replaced by brown, but nonetheless analytic reagent grade, pyridine. For Curve 3 pyridine was replaced by collidine. Curve 4 represents two almost identical curves; in one instance the solution was boiled for 30 minutes and in the other the solution was allowed to stand 5 days at 20° C. between heating and reading. For Curve 5, 0.2 mM. NaOH was added; salts of weak acids gave similar curves. Under many of these altered conditions the blank absorption spectra were also changed. In general, they too tended to become more like the hyperboloid amine curves.

Physical Variables.—An attempt was made to evaluate the effect of several variables in the technique by individually altering them in an otherwise invariant routine. The 417 mμ optical density increased with the length of the heating period to a maximum and thereafter diminished. The time of appearance of this maximum varied inversely with temperature, although its magnitude was independent thereof (Fig. 4). With methylamine it was necessary to warm for one-half hour at 87° C. before the maximum intensity was attained, although

over 90 per cent of the color was found after one-quarter of that time. At this temperature 7 ± 1 minutes was the optimal heating period for the other unbranched aliphatic amines listed in the second part of Table II.

The optical density of the colored ninhydrin-isoamylamine solution was enhanced by time. The increase approximated 5 per cent and was maximal in 1 hour, after which there was a sustained decrease (Fig. 5). This change in absorption spectrum was both quantitative and qualitative. Five days at room temperature converted the double-peaked aliphatic curve to the uninflected hyperboloid type typical of aromatically or heterocyclically substituted amines (Fig. 3).

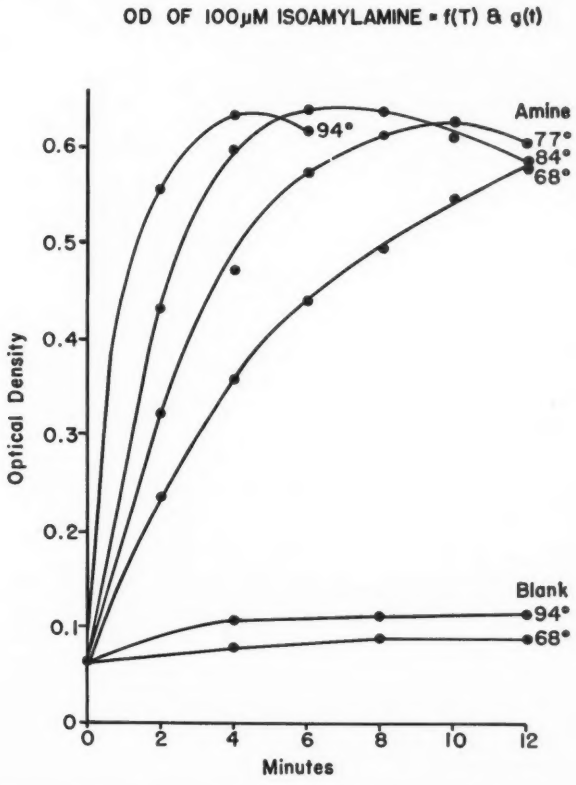

Fig. 4.—Optical density at 417 mμ of ninhydrin-amine complex for 100 micromolar isoamylamine and for the amineless blank solutions as a function of time of heating. Each of the lines represents the centigrade temperature indicated to its right. All solutions were adjusted to their original volume before being read, but loss through evaporation was so severe that the amine curve at 94° C. was terminated at the end of 6 minutes.

Chemical Variables.—Amine, ninhydrin, pyridine, and isopropanol were studied in relation to the reaction; traces of salt and water were added; acid and base content was varied. The plot of *isoamylamine molarity from* 2×10^{-6} *to* 5×10^{-4} versus *optical density at 417 mμ* was a straight line (Fig. 6). The tryptamine and tyramine curves were noticeably nonlinear above 100 micromolar. For methylamine the deviation occurred at a lower concentration and was more marked than for any other amine tested.

Color intensity proved to be a nonlinear function of ninhydrin concentration with the increase continually lessening as the percentage of ninhydrin rose (Fig. 7). Varying the pyridine content caused the strikingly similar changes in the 417 mμ readings indicated in the lower part of the same figure. Pyridine concentration, unlike ninhydrin or amine concentration, affected the shape of the absorption spectrum. The major peak shifted toward the ultraviolet as pyridine decreased.

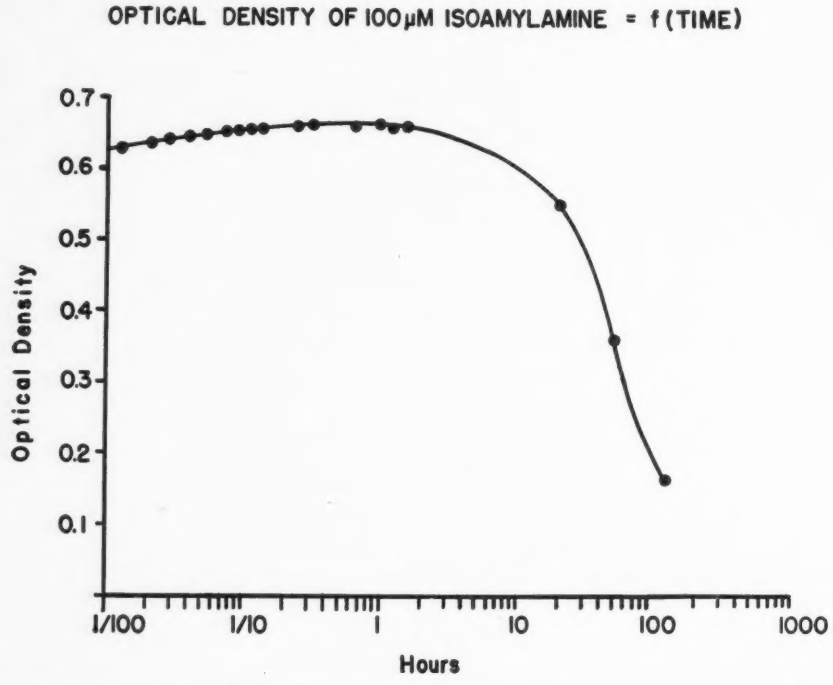

Fig. 5.—Semilog plot of the 417 mμ optical density of ninhydrin-amine complex for a 100 micromolar isoamylamine solution as a function of time between the end of the heating period and reading on the spectrophotometer. The solution was kept at 25° C. during this interval.

When pyridine was replaced by its di- and trimethyl derivatives, lutidine and collidine, the two peaks of the aliphatic amine curve were lowered and the resulting absorption spectrum became hyperboloid (Fig. 3). This change was much more marked for piperidine, with its saturated pyridine ring (Table III, Part 1*).

Many organic liquids were substituted for isopropanol. Ethers, acids, esters, and alcohols produced no qualitative change in the ninhydrin-isoamylamine absorption spectrum, although systematic quantitative variations were considerable. Of a group of miscellaneous substances, carbon tetrachloride and acetone converted the double-peaked blue spectrum to the brown hyperboloid type (Fig. 3). On the other hand, chloroform and benzene proved almost identical with isopropanol (Table III, Part 2).

*The presence of a brown discoloration, not infrequently seen in reagent-grade pyridine, was correlated with bizarre findings. The most important of these was depression of the 417 mμ maximum by as much as a third coupled with conversion of the ninhydrin-amine complex from blue to brown (Fig. 3).

Of interest were trace impurities. Because of its omnipresence, water deserves separate comment. The ninhydrin-isoamylamine complex was sensitive to vestiges of moisture. With no acid and colorless twice redistilled pyridine,* 2½ parts per 1,000 of water increased the 417 mμ optical density 2 per cent over the standard value, while 10 parts of water lowered it more than 10 per cent (Table III, Part 3).

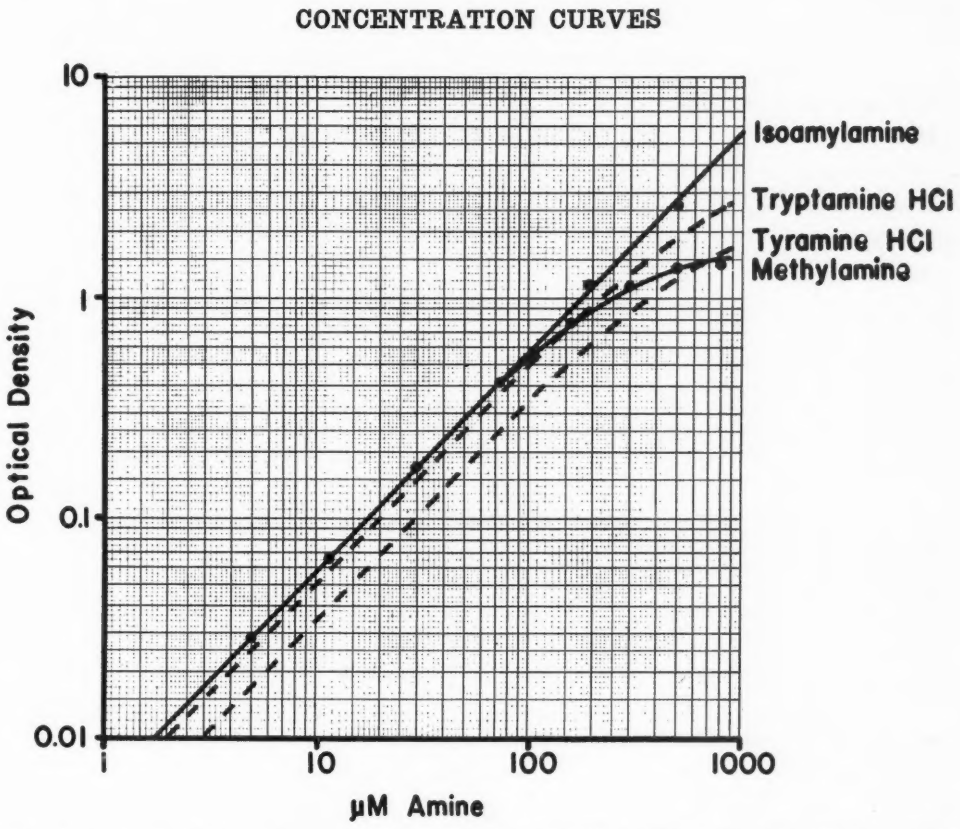

Fig. 6.—Log log plot of amine concentration versus the difference between the 417 mμ optical density of the colored amine solution and that of the amineless blank.

The primary effect of traces of the common inorganic ions found in body fluids was to lessen the optical density of the ninhydrin-isoamylamine solution (Table III, Part 4). Usually there were simultaneous qualitative changes in absorption spectra. These were of three types: First, alkali halides caused a minimal shifting of the 417 mμ peak. Second, all ions tended to displace the 585 mμ maximum to shorter wave lengths. Third, basic media resulting from sufficient solution of the salts of weak acids converted the typical double-peaked absorption spectrum to the hyperboloid type (Fig. 3).

The effect of excess acid or base was considerable on the ninhydrin-isoamylamine reaction. As indicated, alkalinity caused a shift toward the hyper-

*Redistilled dry pyridine (less than 0.1 per cent water as measured by Karl Fischer reagent) was not found superior to entirely colorless samples of commercial analytic reagent pyridine (Mallinckrodt Chemical Company) stored in full, brown, and tightly stoppered bottles.

TABLE III. THE EFFECT OF VARIATIONS IN THE REACTION MEDIA ON THE FORMATION OF NINHYDRIN-ISOAMYLAMINE COMPLEX*

N, 50%	SOLVENT, UP TO 50%	TRACE SUBSTANCE	417 mμ OPTICAL DENSITY†
		Part 1	
Pyridine	Isopropanol		0.581
Lutidine	Isopropanol		0.174‡
Collidine	Isopropanol		0.153‡
Piperidine	Isopropanol		0.007‡
		Part 2	
Pyridine	Isopropanol		0.581
Pyridine	Isobutanol		0.572
Pyridine	Methanol		0.092
Pyridine	Ethanol		0.200
Pyridine	n-Propanol		0.466
Pyridine	n-Butanol		0.696
Pyridine	Formic acid		0.296
Pyridine	Acetic acid		0.381
Pyridine	Propionic acid		0.412
Pyridine	n-Butyric acid		0.211
Pyridine	Ethyl acetate		0.576
Pyridine	Chloroform		0.602
Pyridine	Benzene		0.561
Pyridine	Lactic acid		0.059
Pyridine	Petroleum ether		0.907
Pyridine	Diethyl ether		0.927
Pyridine	Dipropyl ether		0.023‡
Pyridine	Acetone		0.187‡
Pyridine	Carbon tetrachloride		0.062‡
		Part 3	
Pyridine	Isopropanol		0.581
Pyridine	Isopropanol 49.8%	Water ¼%	0.593
Pyridine	Isopropanol 49.5%	Water ½%	0.568
Pyridine	Isopropanol 49.0%	Water 1%	0.517
Pyridine	Isopropanol 48.0%	Water 2%	0.336
Pyridine	Isopropanol 46.0%	Water 4%	0.093
Pyridine	Isopropanol 42.0%	Water 8%	0.073
		Part 4	
Pyridine	Isopropanol		0.581
Pyridine	Isopropanol	$MgCl_2$ 3mM.	0.227‡
Pyridine	Isopropanol	12 mM.	0.166‡
Pyridine	Isopropanol	50 mM.	0.088‡
Pyridine	Isopropanol	200 mM.	0.092‡
Pyridine	Isopropanol	$CaCl_2$ 20 mM.	0.152‡
Pyridine	Isopropanol	NaCl§	0.210 (purple)
Pyridine	Isopropanol	KCl§	0.215 (purple)
Pyridine	Isopropanol	NH_4Cl§	0.621 (purple)
Pyridine	Isopropanol	NaBr§	0.285 (purple)
Pyridine	Isopropanol	NaI§	0.297 (red)
Pyridine	Isopropanol	$NaHCO_3$§	0.346
Pyridine	Isopropanol	Na_2CO_3	0.087‡
Pyridine	Isopropanol	NaAc§	0.152‡
Pyridine	Isopropanol	Na_3PO_4§	0.162‡
Pyridine	Isopropanol	Na_2SO_4§	0.483‡

*Invariant ninhydrin and isoamylamine concentrations of 0.04 per cent and 100 micromolar, respectively, were used.

†Optical densities are those of the solutions minus that of the amineless blank.

‡A brown hyperboloid absorption spectrum is so indicated. Unless otherwise designated the typical blue double-peaked type was present.

§Four milliliters of the isopropanol was saturated by 24 hours' agitation with salt crystals and then was used to develop the color in a total of 10 ml.

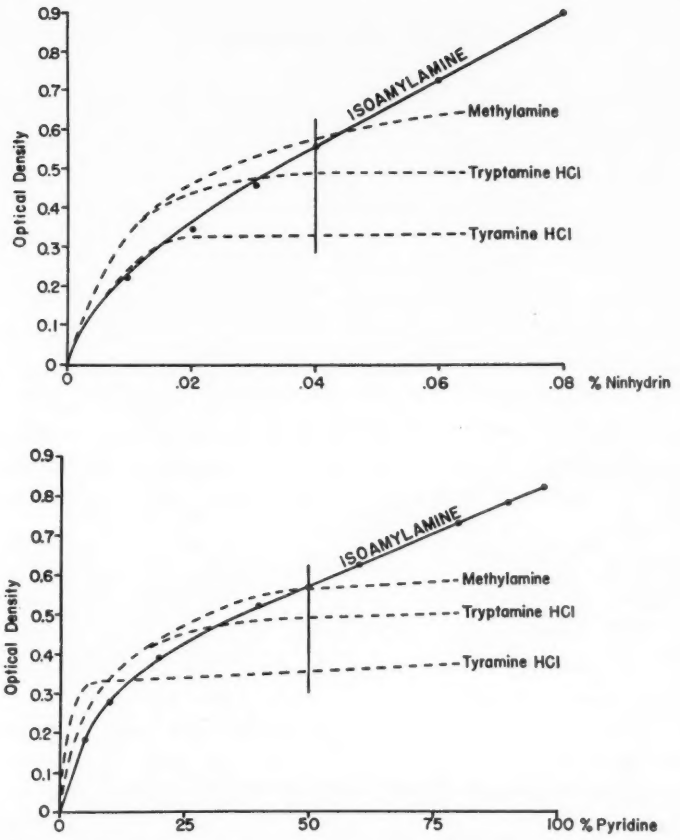

Fig. 7.—The difference between the 417 mμ optical density of ninhydrin-amine complex and that of the amineless blank as a function of ninhydrin and pyridine percentages. All amine concentrations were 100 micromolar. For the upper graph the pyridine was the standard 50 per cent and for the lower graph the ninhydrin was the standard 0.04 per cent, as indicated by the vertical lines.

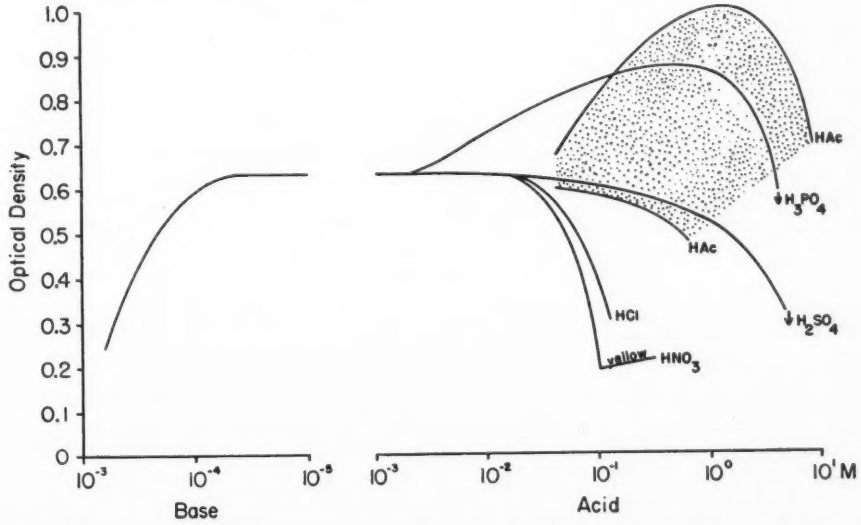

Fig. 8.—Effect of pH on 417 mμ optical density of isoamylamine. Optical density at 417 mμ of ninhydrin-amine complex for 100 micromolar isoamylamine solutions as a function of added acid and base. The curves for all the bases tried were very similar. The varying effects of different acids are indicated. The arrows at the end of the H_2SO_4 and H_3PO_4 curves indicate precipitation. The break in the HNO_3 curve represents a discontinuous color change from blue to yellow. The stippled area between the two *HAc* curves indicates the range over which the readings varied unpredictably in the presence of acetic acid.

boloid type of spectrum. The resultant yellow color was probably due to phenylglyoxal-o-carboxylic acid.[4] Unlike basicity, acidity produced several anomalies, and it was impossible to separate the effect of hydrogen ion from its anion (Fig. 8). Acetic and phosphoric acids were noteworthy in that they enhanced the opacity of the complex, although reproducible results could not be obtained with high concentrations of the former.

Other Amines.—With the standard procedure, straight chain and unsubstituted primary aliphatic amine bases in 100 micromolar concentrations gave very similar optical densities corresponding to molecular extinction coefficients of 5,800 ± 300 (Table II, Part 2). The hydroxyl group in ethanolamine affected the value only slightly. On the other hand, branching close to the amine radical had a profound effect. Isopropylamine had a very low extinction coefficient, isobutylamine a moderately low one, and isoamylamine was typical of the unbranched group.

Aromatically and heterocyclically substituted amines were a more diverse group. The 417 mμ optical density was a less satisfactory index of their concentrations, both from the standpoint of the steep slope of the absorption spectrum in this region and from that of a lower molecular extinction coefficient. For histamine, histamine dihydrochloride, tyramine hydrochloride, tryptamine hydrochloride, and phenylethylamine the molecular extinction coefficient was between 3,000 and 5,000. For the last, however, it was very difficult to get reproducible results, in part because the length of the heating period was uniquely critical.

DISCUSSION AND SUMMARY

The limiting and optimum conditions for determining small amounts of some biologically interesting amines have been presented. One-tenth to one-hundredth of a micromol can easily be measured. The method assays total primary aliphatic amine groups provided they occur on unbranched and unsubstituted carbon chains. For aromatically and heterocyclically substituted amines, estimation is less accurate unless known standards can be used for comparison. Water content, electrolyte concentration, and excess acid or base in the sample to be tested must be within prescribed limits for optimal results. The method has been applied to extracts of urine.[5]

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A SIMPLIFIED METHOD FOR ESTIMATION OF THE EXTRACELLULAR FLUID SPACE

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WITH THE TECHNICAL ASSISTANCE OF BETTY J. HUFFMAN, B.A., AND MARY LOUISE BROSNAHAN, B.A.

IN 1952, Cardozo and Edelman* discussed the problems of measurement of the extracellular fluid volume and presented a method for its determination utilizing the volume of distribution of injected sodium thiosulfate. The validity of the use of the thiosulfate ion is accepted, but the procedure as described by them is complex and time-consuming. We have been able to simplify it greatly by "canceling out" several steps and at the same time to increase the technical precision of the determination.

MATERIALS AND METHODS

A. Reagents.—

10 ml. sealed ampules of sterile approximately 10 per cent $Na_2S_2O_3$ for intravenous use
N/15 H_2SO_4
1 per cent Na_2WO_4
0.1 N KIO_3 (approximate)
0.002 N KIO_3 (approximate) made by diluting the previous 1:50
2 N HCl (approximate)
10 per cent KI (approximate) freshly made
0.1 N $Na_2S_2O_3$ (approximate)
0.001 N $Na_2S_2O_3$ (approximate) made by diluting the previous 1:100
1 per cent starch solution freshly prepared

B. Methods.—

1. Using sterile precautions, place in a clean, sterile 250 ml. Erlenmeyer flask about 130 ml. of sterile approximately 10 per cent sodium thiosulfate solution for intravenous use.†

2. Connect a clean sterile 100 ml. glass stopcock burette to a short length of disposable intravenous tubing and this in turn to a 2-way valve. Rinse the equipment with a few milliliters of the thiosulfate solution and then fill it to near the top of the graduations of the burette, eliminating all air bubbles from the system. Close the glass stopcock and the 2-way valve.

3. Attach an infusion of 5 per cent dextrose in distilled water to the other arm of the 2-way valve.

4. Make a venipuncture in an arm vein and withdraw a 10 ml. sample of "base-line" blood. This should be centrifuged and the serum separated from the clot as soon as possible. All subsequent blood samples should be handled similarly.

5. Attach the 2-way valve to the intravenous needle and allow the dextrose solution to run in slowly for a few minutes to clear the equipment and to check it for leaks.

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*Cardozo, R. H., and Edelman, I. S.: The Volume of Distribution of Sodium Thiosulfate as a Measure of the Extracellular Fluid Space, J. Clin. Invest. 31: 280-290, 1952.

†Supplied in 10 ml. sealed ampules by Winthrop Stearns, Inc.

6. Turn the 2-way valve to the thiosulfate solution. Note the level of the solution in the burette and the time. Then open the glass stopcock and adjust the rate of flow to infuse about 99 ml. of thiosulfate in about 10 minutes.

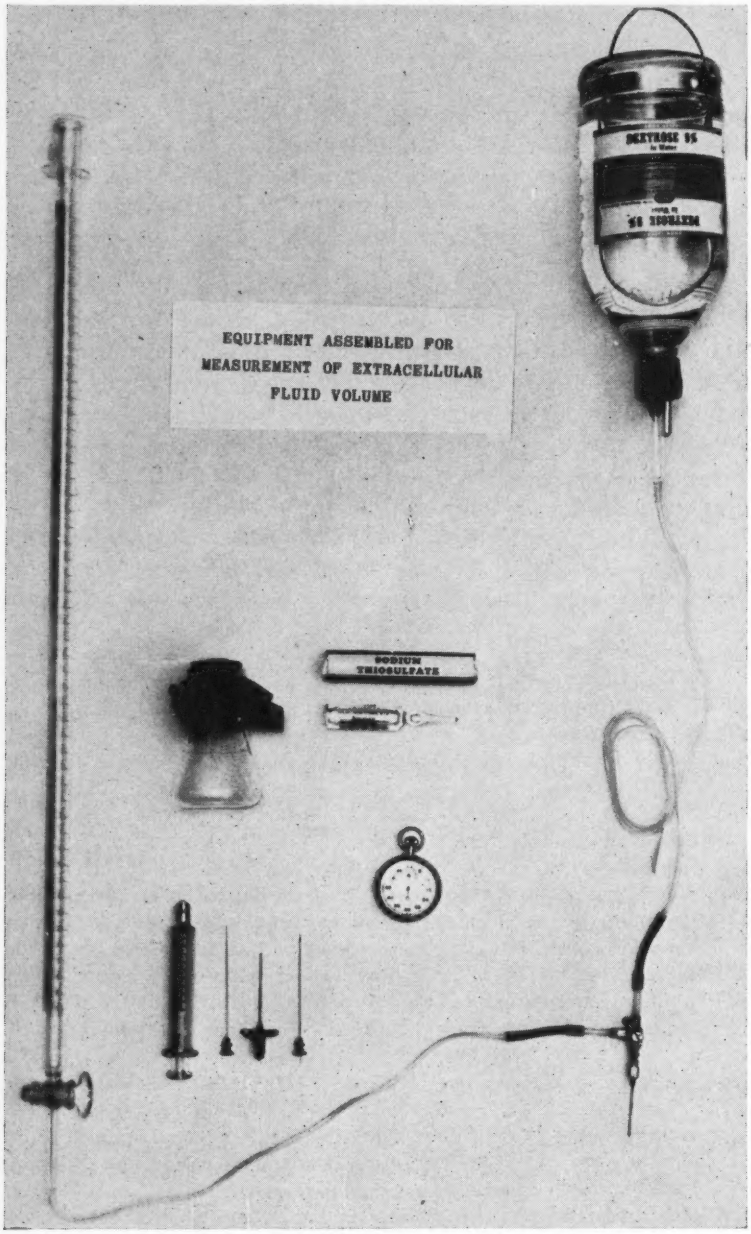

Fig. 1.

7. When the infusion is complete close the glass stopcock, note the time, and turn the 2-way valve back to dextrose solution. Allow about 10 to 20 ml. of this to wash through any thiosulfate remaining in the valve and needle. Remove the apparatus from the vein and note the final level in the burette.

8. Using a vein in the other arm draw 5 ml. samples of blood at approximately 10- to 12-minute intervals for 1 hour. Note the time of beginning and ending the withdrawal of each sample.

9. Zero time is the mid-time of the infusion; sample times are the mid-times of withdrawal.

10. Preparation of samples for titration (run in duplicate): Either serum or heparinized plasma may be used.

Into 50 ml. Erlenmeyer flasks place the following:

	BLANK (ML.)	STANDARD (ML.)	SAMPLE (ML.)
Water	16.0	15.6	16.0
N/15 H_2SO_4	2.0	2.0	2.0
Serum* (base-line)	0.4	0.4	—
Serum* sample	—	—	0.4
1% Na_2WO_4	2.0	2.0	2.0
Standard (1:100 dilution of an aliquot of the same thiosulfate solution infused into the patient)	—	0.4	—

*Serum and tungstate should be added slowly with agitation.

Let stand 10 minutes, transfer to 16 by 150 mm. tubes, centrifuge 10 minutes at high speed, and decant the clear supernate.

11. Titration of protein-free solutions:

To 5.0 ml. approximately 0.002 N KIO_3 in a 50 ml. Erlenmeyer flask add

8.0 ml. protein-free solution,

1.0 ml. approximately 2 N HCl.

Let stand 5, and not more than 10, minutes. Then add

1.0 ml. approximately 10 per cent KI and titrate immediately with

0.001 N $Na_2S_2O_3$ (approximate) solution until the iodine color has almost disappeared. Add 2 or 3 drops 1 per cent starch solution as indicator and titrate to the disappearance of the blue color.

12. Calculations:

(a) Blank titration − sample titration = ml. thiosulfate for sample.

(b) Blank titration − standard titration = ml. thiosulfate for standard.

(c) Plot ml. thiosulfate solution for samples on a semilogarithmic paper against time.

(d) Extrapolate the late slope (after 20 minutes) to zero time.

(e) $\frac{\text{ml. thiosulfate for standard}}{\text{ml. thiosulfate for a sample at 0 time}} \times 100 \times \text{ml. thiosulfate infused} = \text{extracellular fluid volume.}$

DISCUSSION

The measurement of the volume of infused thiosulfate with a burette, in the manner described, eliminates the necessity (1) for determining the density of the infused solution, (2) for determining the quantity administered by the difference in the weight of the container, and (3) for quantitative transfers including rinsing of the infusion flask. Since the infused thiosulfate and the laboratory standard are both aliquots of the same sample, it is enough to measure their relative concentrations directly without the need to determine their absolute concentrations. This makes it unnecessary to standardize accurately any of the reagents.

In the determination of extracellular fluid volume, the performance error of the procedure itself is considerably smaller than the inaccuracies due to biologic factors, such as unequal distribution of infused thiosulfate or variation between samples, etc. The suggested modifications favorably affect the performance error but in no way alter the biologic error. The *technical* accuracy of the procedure, then, depends on the precision with which a given volume of thiosulfate solution can be delivered to the patient and the precision with which the final titrations are made. If spillage and air bubbles are avoided, the former corresponds to the accuracy with which two levels can be read on a 100 ml. burette. We believe that with this simplification the estimation of extracellular fluid volume is within the practical reach of most clinical laboratories.

SUMMARY

A simplification of method for the determination of extracellular fluid volume with thiosulfate ion is presented. Accurate delivery of a measured volume of sodium thiosulfate solution is made possible with a sterile burette and disposable tubing. The use of a diluted aliquot of the infused solution as a reference standard eliminates the need for determining the absolute amounts of thiosulfate delivered or present in the serum.

A SIMPLIFIED DYE METHOD FOR ESTIMATING PLASMA VOLUME

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KANSAS CITY, MO.
WITH THE TECHNICAL ASSISTANCE OF BETTY J. HUFFMAN, B. A., AND
MARY LOUISE BROSNAHAN, B.A.

DESPITE the current popularity and possible advantages of radioactive tracer methods for measuring plasma volume, these methods are not readily available to all who wish to use them on patients. The Evans blue (T-1824) method is, therefore, still widely used. It has been shown to be at least as valid as methods using radioactive iodinated human serum albumin.[3, 5, 8] However, as commonly described[1, 2, 6, 7] and used, the dye technique is cumbersome and subject to many sources of performance error. By applying some of the principles used in radioactive tracer techniques, we have been able to simplify greatly the procedure and at the same time to eliminate or minimize several sources of error.

METHOD

1. Approximately 5 ml. of a 0.5 per cent aqueous solution of Evans blue dye (as supplied in sterile ampules*) is diluted in approximately 20 ml. of sterile normal saline solution for intravenous use contained in its original rubber-capped vial.

2. Exactly 20.0 ml. of this diluted dye is withdrawn into a sterile syringe which has been calibrated† previously to deliver 20.0 ml. with an accuracy of ± 1 per cent. A small bubble of air is drawn into the lumen of the needle adapter of the syringe after the dye to minimize chances of dye loss when the syringe is later connected to the needle.

3. A 20 gauge needle is inserted into an arm vein for withdrawal of a 10 ml. sample of blood and for the administration of the dye. This blood is delivered into a tube containing dry anticoagulant, either heparin or potassium–ammonium oxalate mixture.

4. An aliquot of this ''base-line'' blood is used for hematocrit determination by means of a Wintrobe tube. Centrifugation is carried out for 30 minutes at 3,000 rpm at a radius of 20 cm. (to the bottom of the tube). This creates a centrifugal force of slightly more than 2,000 times gravity and results in an error of hematocrit due to trapping of plasma in the red cell column of less than 2 per cent of the whole blood volume.[9]

5. After withdrawal of the ''base-line'' blood, the syringe containing dye is inserted into the needle and the dye is injected. The times of beginning and of finishing the injection are noted. ''Zero time'' is the mid-time of injection. The lower end of the syringe and the needle are rinsed twice by withdrawing and reinjecting 1 ml. of blood. The needle is then withdrawn from the vein.

6. Samples of venous blood are withdrawn from the opposite arm at approximately 10-minute intervals for 1 hour. The beginning and ending times of each sampling are accurately noted, and the mid-time is calculated. The blood is preserved in tubes containing anticoagulant.

7. All blood samples are centrifuged and the plasma separated from the cells. Any plasma sample with marked hemolysis is then discarded. A correction may be made for minor degrees of hemolysis.

From the Research Laboratories of the Veterans Administration Hospital, Kansas City, Mo., and the Department of Surgery of the University of Kansas School of Medicine.

We are indebted to David V. Cohn, Ph.D., for perfection of the correction for hemolysis.
Received for publication March 14, 1955.

*Supplied by Wm. R. Warner, New York.

†The syringes can be calibrated by delivery of mercury into a weighing vessel or by delivery of water into a standard burette.

8. A standard solution is then prepared from an aliquot of the same diluted dye which was used for injection. Five-tenths milliliter of this diluted dye is further diluted to 10.0 ml. Of this solution 0.2 ml. is added to 1.8 ml. of the plasma from the "base-line" sample.

9. Each of the dyed plasma samples is prepared by adding 0.2 ml. of normal saline solution to 1.8 ml. of plasma.

10. The optical densities of standard and samples are determined in a Coleman Junior spectrophotometer at 620 mμ, using as a blank 0.2 ml. normal saline solution in 1.8 ml. of base-line plasma. If recognizable hemolysis is present in any sample, a correction may be made by reading its optical density at 540 mμ as well as at 620 mμ and applying the following formula:

$$OD_{\text{Dye 620 corrected}} = \frac{OD_{\text{Total 620}} - 0.1\ OD_{\text{Dye 540}}}{0.944}.$$

11. The values for optical density of the dyed plasma samples are plotted against time on semilogarithmic paper. The late or "disappearance" slope is then extrapolated to time zero and the corrected optical density of the plasma diluted dye is read.

12. The plasma volume equals the volume of dye injected times the dilution of the standard times the reciprocal of the dilution of the dye plasma times the ratio of the optical density of the standard to the optical density of the plasma-diluted dye.

$$PV = 20 \times 200 \times 0.9 \times \frac{OD\ Std}{OD\ Pl}\ \text{(corrected)}$$

DISCUSSION

The usual directions for the use of Evans blue involve weighing the syringe before and after delivery of the dye, determination of the specific gravity of the dye, and calculation of the quantity delivered. This may increase somewhat the accuracy of delivery of 5 ml. amounts but at the expense of adding several steps requiring precision. The use of 20 ml. quantities and calibrated syringes

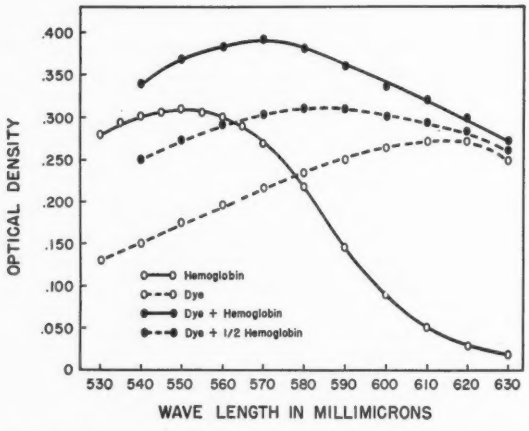

Fig. 1.—Absorption spectra of T-1824 (dye) and hemoglobin.

allows delivery of a known quantity with greater ease and equal accuracy. The use of aliquots from a single approximate dilution of dye, for both preparation of the standard and injection into the patient, eliminates the need to standardize each batch of dye or to prepare accurate dilutions. It is, after all, necessary to know only the ratio of concentrations of dye in the injected material and in the plasma. Problems in measurement of the dye are raised by the fact that the optical density is not the same when dye is dissolved in water and in various

plasmas.[4] Such problems are eliminated by this method in which the blank, the standard, and the samples contain identical quantities of the patient's own plasma and saline as solvents. While estimations of plasma volume using a single 10-minute sample can be made in normal persons with reasonable accuracy, often they do not suffice in patients with altered circulation.[2] We have seldom found the optical density of the 10-minute sample to lie on the disappearance curve. The use of serial samples to establish the disappearance slope makes less likely the invalidation of the determination by a random error.

The dyed plasma samples frequently contain varying amounts of hemoglobin despite careful technique. This introduces a real error, but correction can be made by determining the optical density at the range of the optimum absorption of hemoglobin as well as at the optimum absorption of T-1824. Studies were made by using the same solvent as in actual determinations, i.e., 1.8 ml. plasma and 0.2 ml. saline. Solutions of dye, free of hemoglobin, show a plateau of absorption from 610 mμ to 620 mμ. When hemoglobin is added, the absorption over the plateau is minimally increased at 620 mμ. This is, therefore, the optimum for dye determination. Hemoglobin absorbs optimally in the region of 540 mμ and here the dye absorbs little.

The correction for hemoglobin can be derived as follows*:

TERMS

OD_{D540} = Optical density of plasma containing dye only, at 540 mμ
OD_{D620} = Optical density of plasma containing dye only, at 620 mμ
OD_{H540} = Optical density of plasma containing hemoglobin only, at 540 mμ
OD_{H620} = Optical density of plasma containing hemoglobin only, at 620 mμ
OD_{T540} = Optical density of plasma containing dye plus hemoglobin at 540 mμ
OD_{T620} = Optical density of plasma containing dye plus hemoglobin at 620 mμ
$K_H = 0.100$
$K_D = 0.562$ (to be empirically determined in each laboratory)

PRIMARY RELATIONSHIPS

$$OD_{T540} = OD_{D540} + OD_{H540} \quad (1)$$

$$OD_{T620} = OD_{D620} + OD_{H620} \quad (2)$$

$$\frac{OD_{D540}}{OD_{D620}} = K_D \text{ or } OD_{D540} = K_D \times OD_{D620} \quad (3)$$

$$\frac{OD_{H620}}{OD_{H540}} = K_H \text{ or } OD_{H620} = K_H \times OD_{H540} \quad (4)$$

DERIVATION

Subst. (3) in (1) $OD_{T540} = [K_D \times OD_{D620}] + OD_{H540}$ (5)

Subst. (4) in (2) $OD_{T620} = OD_{D620} + [K_H \times OD_{H540}]$ (6)

Subst. (1) in (6) $OD_{T620} = OD_{D620} + K_H [OD_{T540} - OD_{D540}]$ (7)

Subst. (3) in (7) $OD_{T620} = OD_{D620} + K_H [OD_{T540} - K_D OD_{D620}]$ (8)

Removing [ ] $OD_{T620} = OD_{D620} + K_H OD_{T540} - K_H K_D OD_{D620}$ (9)

Transposing $OD_{D620} - K_H K_D OD_{D620} = OD_{T620} - K_H OD_{T540}$ (10)

CORRECTION EQUATION

Factoring $OD_{D620} [1 - K_H K_D] = OD_{T620} - K_H OD_{T540}$ (11)

Solving for OD_{D620} $$OD_{D620} = \frac{OD_{T620} - K_H OD_{T540}}{1 - K_H K_D} \quad (12)$$

*This differs from the correction described by Chinard[2] only in the definition of K_D and in the use of 540 mμ rather than 550 mμ.

SUMMARY

A simplified method for estimating plasma volume with Evans blue dye is presented. The procedure is performed on aliquots of diluted dye, eliminating entirely the need to determine absolute amounts of dye. Problems due to differing optical densities of T-1824 in different solvents are eliminated by preparing all samples in a solvent of constant composition using the patient's own serum. A method for correcting optical densities for hemolysis is derived.

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Erratum

In the Progress Report entitled "Primary Aldosteronism" by Dr. Jerome W. Conn, which appeared on page 661 of the April issue of the JOURNAL, the first sentence should read as follows: "On Oct. 29, 1954, we first described a new clinical syndrome which we named primary aldosteronism."

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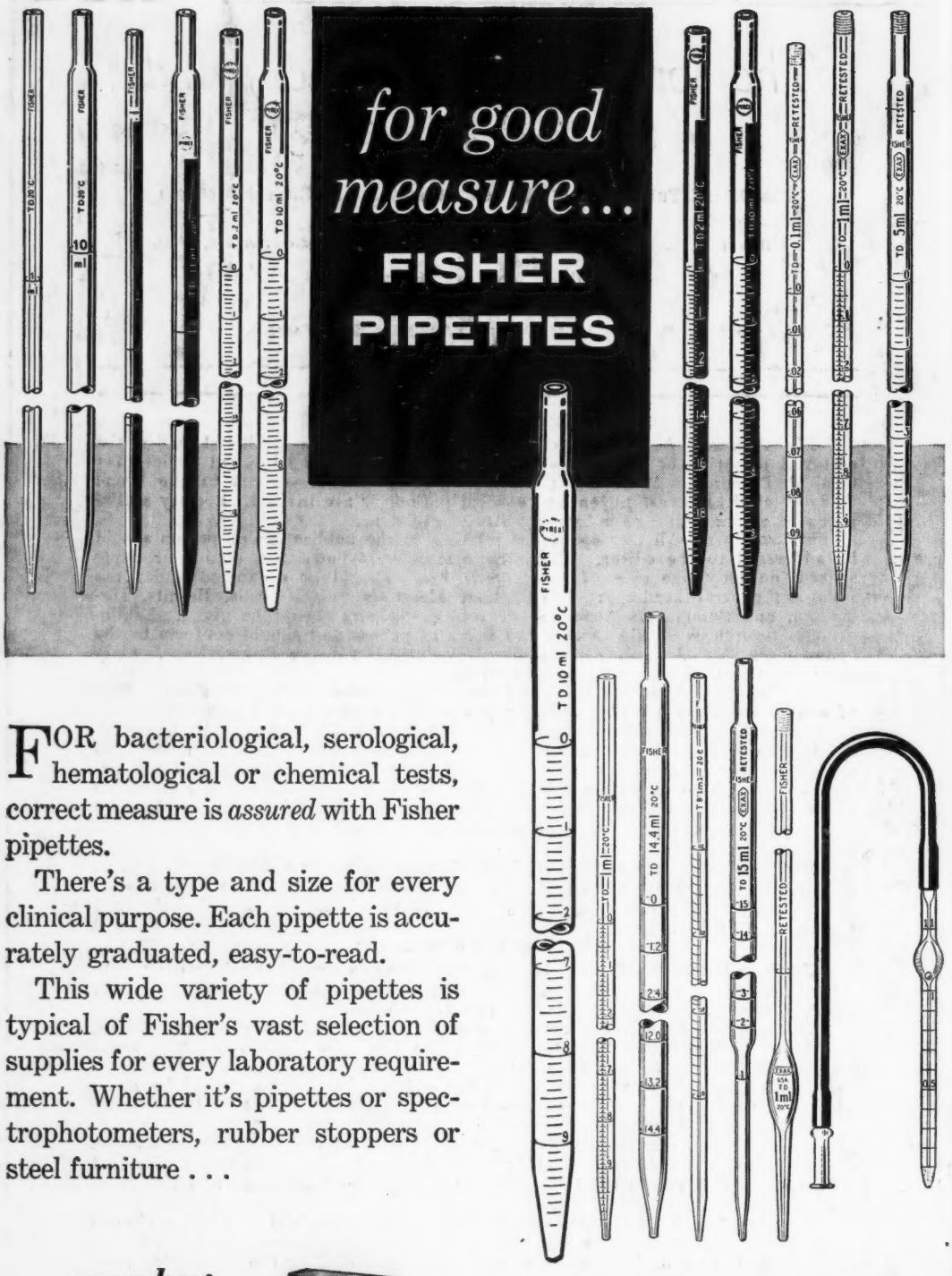

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